# THE ROLE OF ANDROGENS IN MALE PREGNANCY AND FEMALE COMPETITIVE BEHAVIOR IN A SEX ROLE REVERSED PIPEFISH

A Dissertation

by

SUNNY KAY SCOBELL

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2011

Major Subject: Biology

The Role of Androgens in Male Pregnancy and Female Competitive Behavior in a Sex Role Reversed Pipefish

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December 2011

Major Subject: Biology

# ABSTRACT

The Role of Androgens in Male Pregnancy and Female Competitive Behavior in a Sex Role Reversed Pipefish. (December 2011)

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Co-Chairs of Advisory Committee: Dr. Adam G. Jones
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The sex-role reversal and male pregnancy found in syngnathids are highly unusual traits in vertebrates. Reproductive hormones likely influence development and regulation of these traits. However, very few studies have examined the underlying hormonal mechanisms that mediate female competitive behavior and male pregnancy. New methodologies and better husbandry practices have made such studies more feasible in recent years. Research on a relatively small number of species has suggested that androgens are likely regulators of spermatogenesis and the development of the male brood pouch prior to pregnancy. Androgens are also potential candidates for mediating sex-role reversed behavior in female syngnathids. The goal of this dissertation was to examine the role of androgens in the male reproductive cycle and female intrasexual competitive behavior in the sex-role reversed Gulf pipefish, *Syngnathus scovelli*.

From review of the literature, I developed a model for the hormonal regulation of the male reproductive cycle in seahorses. I predicted that androgens would be low during the early stages of pregnancy and increase during the end of pregnancy as males go

through another cycle of spermatogenesis in preparation for the next mating event. My study of 11-ketotestosterone and testis mass across the reproductive cycle in male *S. scovelli* supported this model. I also conducted several studies on the role of androgens in female competitive behavior. I determined that treatment with 11-ketotestosterone the evening prior to an intrasexual interaction resulted in an increase in competitive behavior in large over small test females. Conversely, treatment with 11-ketotestosterone one hour prior to an intrasexual interaction resulted in a decrease in competitive behavior in large over small females when stimulus female behavior was controlled. A comparative study of competitive and courtship behavior in *S. scovelli* and the closely related *S. floridae* suggested that sexual selection has affected competitive and courtship behavior in both males and females of these species. The diversity of reproductive patterns exhibited by syngnathids suggests that they will provide a unique opportunity to assess how hormonal regulation of reproductive behavior and function has evolved within this lineage.

# DEDICATION

To my mother, father, and sister

You are my constant source of strength, support, and inspiration

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# NOMENCLATURE

| | |
|---|---|
| 17,20-P | 17α,20β-dihydroxy-4-pregnen-3-one |
| 17β-HSD | 17β-Hydroxysteroid dehydrogenase |
| 11β-HSD | 11β-Hydroxysteroid dehydrogenase |
| ACTH | Adrenocorticotropic hormone |
| AVP | Arginine vasopressin |
| AVT | Arginine vasotocin |
| $E_2$ | Estradiol |
| $EE_2$ | Ethinylestradiol |
| FSH | Follicle stimulating hormone |
| GnRH | Gonadotropin releasing hormone |
| IT | Isotocin |
| KA | Ketoandrostenedione |
| KT | 11-ketotestosterone |
| LH | Luteinizing hormone |
| MIS | Maturation inducing steroid |
| OHA | 11β-hydroxyandrostenedione |
| OT | Oxytocin |
| PRL | Prolactin |
| T | Testosterone |
| TP | Testosterone proprionate |

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# CHAPTER I

# INTRODUCTION AND

# LITERATURE REVIEW*

## Introduction

Throughout most of the animal kingdom, males have elaborate secondary sexual traits and compete to mate with females, whereas females are more active in mate choice. A large body of literature has examined how sexual selection has helped to shape these traits from an ultimate to a proximate level. A few species do not follow this norm: in those, females with elaborate traits compete to mate with choosy males. These sex-role reversed species challenge the assumptions of sexual selection theory for if the assumptions are correct, they should be supported in both conventional and sex-role reversed mating systems. Sex-role reversed species are particularly challenging when it comes to understanding the physiology that underlies a reversal of behavioral patterns in males and females. In vertebrates of species with conventional mating systems, gonadal hormones shape much of the reproductive behavior within a sex. For sex-role reversal to be an evolutionary stable strategy, these species must have physiological mechanisms that support both behavioral and reproductive function. Within a lineage, sex-role reversed traits may have evolved by accessing molecular and hormonal mechanisms that

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This dissertation follows the style of *Hormones and Behavior*.

*The Literature review and Future directions sections of this chapter have been published previously: Scobell, S.K., Mackenzie, D.S., 2011. Reproductive endocrinology of Syngnathidae. J. Fish Biol. 78, 1662-1680.

evolved in the opposite sex or they may have evolved through the development of physiological pathways that produce a convergent phenotype (Ketterson, 2007).

All species in the teleost Family Syngnathidae have evolved male pregnancy; the specialized tissues of the brood pouch have been implicated in aeration, osmoregulation, nutrient provisioning, and immune defense of offspring (Scobell and Mackenzie, 2011). The Family also contains species with some of the most elaborate female ornamentation observed in vertebrates (Berglund and Rosenqvist, 2003). Some species exhibit extreme polyandry and sex-role reversed mating behavior, whereas others, despite male pregnancy, have conventional sex roles (Jones and Avise, 2001a). This Family provides the opportunity to tease apart the physiological mechanisms that selection has acted upon to produce such a variety of morphological and behavioral traits.

Because teleost fish exhibit a high degree of sexual plasticity (Desjardins and Fernald, 2009; Godwin, 2010), I hypothesized that sex-role reversed behavior in female syngnathids evolved through a mechanism by which females tapped into an ancestral male behavioral network. As such, I predicted that androgens would mediate these behaviors as they mediate the development and maintenance of many behavioral traits in males of conventional species. In addition, due to their crucial role in spermatogenesis, I also predicted that androgens would mediate reproduction in male syngnathids. I used the polyandrous, sex-role reversed Gulf pipefish, *Syngnathus scovelli* (Fig. 1.1), as my model species to investigate this hypothesis.

Photo courtesy of Nick Ratterman

Fig. 1.1. A female Gulf pipefish, *Syngnathus scovelli*, in the ‘pose’ posture while displaying the temporary ornament (a darkening of the melanistic areas of the trunk, tail, and dorsal fin), which highlights the permanent ornament (a silver bar on each ring of the trunk).

*Study species*

The Gulf pipefish is sex-role reversed and highly polyandrous (Jones et al., 2001b). Females have a higher standardized variance in mating success (Jones et al., 2001b) and a higher potential reproductive rate (PRR) than males (1.95:1.00 females:males; (Scobell *et al.*, 2009). Females are typically larger than males and have a deeply keeled abdomen (Jones and Avise, 2001a). Females have a permanent sexually dimorphic ornament, a silvery bar on each bony ring of the trunk (Reid, 1954), and display a temporary ornament during courtship and competition (Scobell *et al.*, *In prep*). Females also have a larger dorsal fin than males (Brown, 1972; Jones and Avise, 2001a) that darkens along with the trunk during the temporary ornament display (SKS personal observation). In the field, mated females are more ornamented, and have a larger mean snout-vent length, body depth, and body mass than unmated females (Jones et al., 2001b). In the lab, females compete in the presence and absence of males (Scobell *et al.*, *In prep*) and are more active in courtship than males (SKS personal observation).

*Female intrasexual competition in* S. scovelli

When females first notice each other, one or both females often display their temporary ornament. This involves a rapid transformation from the typical cryptic brown or green coloration to a contrasting black-and-white banding pattern down the length of the body (Fig. 1.2). A small flap of skin, similar to the skin fold of *Nerophis ophidion*

(Rosenqvist, 1990), darkens during the display, which highlights the keel of the abdomen. The darkened dorsal fin helps to delineate the dorsal part of the trunk, and the vertical bands accentuate the silver bars on the lateral side of the trunk. As the females approach each other, usually horizontally, they align the sides of their bodies laterally and one or both females pose while displaying the temporary ornament. These lateral displays progress into vertical dancing, in which the females parallel each other's movements. During these lateral displays, one female often leans the trunk of her body toward the other female. During intense interactions, the females twitch, violently shaking the entire body for a few seconds.

*Courtship and copulation in* S. scovelli

The behaviors exhibited during courtship are very similar to those of competition. Female *S. scovelli* are usually the first to initiate courtship. Shortly after becoming aware of the male, the female poses and begins to display the temporary ornament. If the male is receptive, he responds by lifting his head similar to the pose in females, but without an arched back (males never display a temporary ornament). The female then moves from a pose into a dance, which is a short swim in the vertical position. If the male follows with a dance, the female proceeds to lean toward the male and then often twitches. Males frequently respond by twitching themselves. Females usually perform repeated rounds of leaning and twitching toward the male while moving vertically up and down in the tank. During the final dance, the female increases her twitching before wrapping her tail

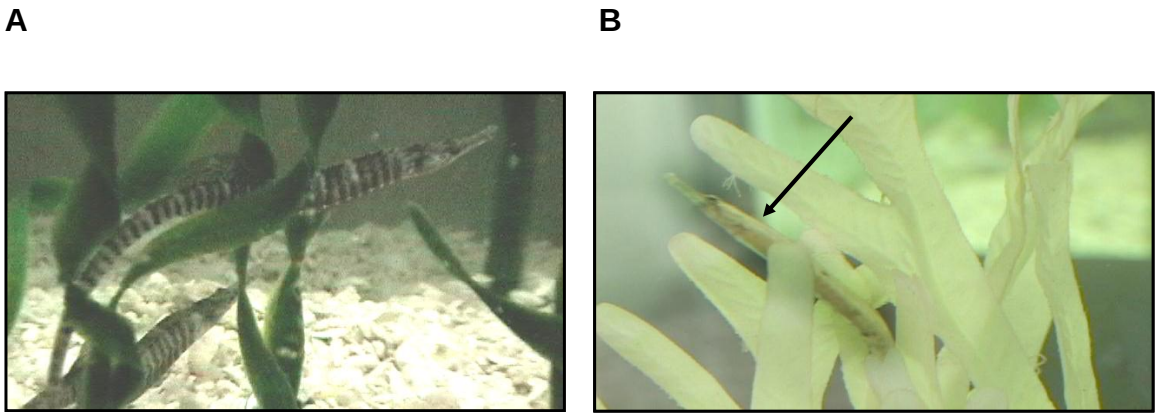



Fig. 1.2. (A) Two female *S. scovelli* displaying the temporary ornament during an intrasexual competition. (B) A single *S. scovelli* female with the typical cryptic coloration (arrow).

around that of the male. This quick move is followed by the female placing her ovipositor (an out-pocketing of the urogenital tract) into the top of the male's pouch and transferring the eggs. The male and female spiral downward around each other in the water column during copulation. One of the pair will break away from the copulation, which ends egg transfer. Then, the male swims immediately down to the bottom of the tank where he undulates his pouch against the substrate, presumably fertilizing the eggs at this time. The time between the onset of courtship and the completion of copulation in *S. scovelli* can be just a minute or two.

*Chapter outline*

In **CHAPTER II,** I tested the predictions of the model detailed in **CHAPTER I** regarding androgen mediation of the male reproductive cycle in syngnathids. I measured plasma levels of the androgen 11-ketotestosterone in field-caught male and female *S. scovelli* and correlated them with morphological measures and gonad mass in both sexes and with respect to reproductive state in males.

In **CHAPTER III,** I injected females with the androgens 11-ketotestosterone and 11β-hydroxyandrostenedione, and the glucocorticoid cortisol to determine their effects on female intrasexual competition in *S. scovelli*.

In **CHAPTER IV,** I injected females with the androgens 11-ketotestosterone immediately prior to the trial to determine their effects on female intrasexual competition in *S. scovelli*.

In **CHAPTER V**, I synthesize the results from the previous chapters with respect to the evolution of male pregnancy and female intrasexual competition in *S. scovelli* and I suggest future directions for endocrine studies in syngnathids.

**Literature review**

*"Thus our Sea-horse, though anomalous in form and habit, has beauty united with its strange features, and grace with its eccentricity." –Samuel Lockwood*

Naturalists and researchers have been interested in the unique adaptations of syngnathid fish for more than one hundred years. Lockwood (1867) was the first to suggest that seahorse *pater-maters* (father-mothers) provision their young in the brood pouch, but could not explain the physiology of this phenomenon. Since then, studies in diverse teleost fish species have established that hormones play a central role in regulating all aspects of fish reproductive biology, including gonadal differentiation, gametogenesis, development of sexual characters, and expression of reproductive behaviors (Lubzens et al., 2010; Munakata and Kobayashi, 2010; Schulz et al., 2010; Zohar et al., 2010). Whereas much of this research has focused on fish species of economic importance, the techniques developed to characterize hormone synthesis, secretion, and receptor function are broadly applicable to other teleost species. It is thus surprising that relatively little is known about the endocrinology of syngnathids, a family that has such intriguing reproductive specializations as male pregnancy, sex-role

reversal, and a diversity of mating systems. Syngnathids should be of keen interest as a taxon that may contribute to the understanding of the role of hormones in the evolution of diverse traits, a fundamental question in comparative endocrinology (Denver *et al.*, 2009). However, there are challenges to performing traditional endocrine studies in these fishes.

Due to the small size of most syngnathids and the difficulties of maintaining them in captivity, it has been challenging to apply traditional methods for studying hormone function, including direct measurement of hormones in plasma or tissues, endocrine gland extirpation-replacement, and *in vitro* competitive binding studies of receptor function. One of the greatest challenges is the small plasma volume of most species. Typical plasma samples from larger syngnathids can range from 60-200 µl, but plasma samples in smaller syngnathids range from 10-20 µl (Mayer *et al.*, 1993; Poortenaar *et al.*, 2004). This restricts direct measurement of circulating hormones in individual animals to only the most sensitive assay techniques. In many species, pooling plasma samples from several individuals is necessary and information on individual variation in hormone levels is lost (Mayer *et al.*, 1993). Plasma sampling in smaller species is usually terminal, precluding repeated measurement of hormones and necessitating increased sample sizes. In most species of syngnathids, the primary endogenous steroid hormones have yet to be identified. Likewise, validated techniques for measurement of homologous protein hormones have not been developed, and established assays for many structurally conserved hormones (e.g., arginine vasotocin, AVT; glucocorticoids; thyroid hormones; or catecholamines) have only recently begun to be applied (Ripley &

Foran, 2009a, 2010). This lack of information on the identity or circulating concentrations of endogenous hormones impedes hormone administration studies, as appropriate homologous hormones and physiological doses for administration are not known.

Despite these obstacles, endocrine studies in a limited number of syngnathid species have addressed some of the most intriguing questions in this field. In this review, the literature on hormone studies of reproductive biology in syngnathids is summarized, focusing on well-established categories of fish reproductive endocrinological investigation (basal hormone levels during reproductive cycles, hormonal regulation of behaviour, endocrine disruption), as well as aspects unique to syngnathids (brood pouch development, male pregnancy). The methodological problems associated with syngnathid endocrinology are addressed and recent techniques that may help solve these problems are suggested. Finally, areas are proposed for future endocrine studies in syngnathids that are likely to be fruitful once technical difficulties have been overcome.

## Hormones in Syngnathids

### *Reproductive cycles*

Reproductive cycles in fish are regulated by the hypothalamic-pituitary-gonadal (HPG) axis in a manner similar to that of most vertebrates. In both sexes, hypothalamic gonadotropin-releasing hormone (GnRH) stimulates the release of the pituitary

gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH) into the circulation. In males, FSH and LH stimulate spermatogenesis and final sperm maturation, respectively (Knapp and Carlisle, 2011b). These gonadotropins also stimulate testicular interstitial (Leydig) cells to produce androgens (Schulz *et al.*, 2010), predominantly testosterone (T) and 11-ketotestosterone (11-KT) in fish. Androgens in turn promote spermatogenesis and development of secondary sexual characters in males. Progestins produced in the testis under gonadotropin control appear to function as gamete maturation-inducing steroids (MIS), promoting final maturation of spermatozoa and spermiogenesis (Schulz *et al.*, 2010; Knapp & Carlisle, 2011). In females, gonadotropins bind to their respective receptors in the ovary, promoting follicle growth and oocyte development partly through stimulation of production of T in the theca cells and estradiol ($E_2$) (via aromatization of T) in the granulosa cells (Lubzens *et al.*, 2010). These steroids have intraovarian functions in promoting follicle development as well as peripheral actions in stimulating vitellogenin production, secondary sexual characters, and behavior. During final oocyte maturation, LH stimulates the production of an MIS, identified as 17α,20β-dihydroxy-4-pregnen-3-one (17,20-P) in many teleost species (Lubzens *et al.*, 2010). Ovulation in teleosts is induced by an LH surge that can be followed by the release of ovarian prostaglandins (Munakata and Kobayashi, 2010), which can act both as a hormone and a pheromone to induce spawning behavior (Stacey, 2011). Currently, the literature on syngnathid endocrinology is limited to a small number of studies (Table 1.1 & 1.2), generally focused on reproductive steroid hormone

measurement or administration; no information exists on the function of GnRH, FSH, LH, or prostaglandins in syngnathids.

Investigations of hormone function in fish traditionally begin with histological examination of endocrine glands and their target tissues, often coupled with measurement of circulating hormone levels. Table 1.1 summarizes the results of descriptive endocrine studies for syngnathids. In comparison to several other teleost orders, little is known about baseline reproductive endocrine function in syngnathids. The most detailed study was undertaken by Boisseau (1967a), utilizing the long-snouted seahorse, *H. guttulatus* Cuvier, and the short-snouted seahorse, *H. hippocampus* (L.). In a remarkably extensive series of studies, the progress of normal gonadal and brood pouch development was characterized, techniques for hypophysectomy and castration were developed, and the effects of steroid and pituitary hormone administration on brood pouch development, pregnancy, and parturition were examined. In these seahorses, the Leydig cells underwent a distinct seasonal cycle of activation, which suggested a prolonged period of androgen synthesis that was maximal during proliferation of spermatocytes and development of the brood pouch, but terminated prior to the appearance of spermatozoa and mating, and was suppressed during gestation (Boisseau, 1967a). In contrast to androgens, a syngnathid MIS may only be present in the circulation of males for very brief periods immediately preceding spawning, as the production of mature spermatozoa in several species seems to be halted until stimulated by courtship behavior (Kornienko and Drosdov, 1999; Van Look et al., 2007; Watanabe et al., 2000). Boisseau (1967a) also observed cycles of activity (via histological indices)

in the pituitary-adrenal axis suggesting glucocorticoid involvement in breeding and brood pouch development.

Only a single study has reported circulating levels of reproductive steroid hormones in male syngnathids. Mayer *et al.* (1993) detected five different androgens, as well as estrogen, in pregnant and non-pregnant mature males of the broadnosed pipefish, *Syngnathus typhle* L., the greater pipefish, *S. acus* L., and the straightnosed pipefish, *Nerophis ophidion* (L.). This latter study confirmed a decline in androgen production from the pre-pregnancy to the pregnancy period, and also identified the rarely studied 11β-hydroxyandrostenedione (OHA) as the predominant androgen. This underscores the need for a thorough characterization of the identity of endogenous reproductive steroids prior to undertaking comprehensive studies of steroid regulation of spermatogenesis, male pregnancy, and reproductive behavior.

As with males, little information is available on baseline levels of female syngnathid reproductive hormones. Mayer *et al.* (1993) detected $E_2$ in two species and up to five different androgens in three species of gravid female pipefish, *S. typhle, S. acus*, and *N. ophidion*. Poortenaar *et al.* (2004) found that plasma T was highest in female big belly seahorses, *H. abdominalis* Lesson, with oocytes in the previtellogenic stage. Androgens, including T, have been implicated in the promotion of growth of oocytes (reviewed in Urbatzka *et al.*, 2011), so it is not surprising that substantial androgen was found in females in both of these studies. However, Poortenaar *et al.* (2004) were unable to detect significant changes in $E_2$ levels across previtellogenic, vitellogenic, and maturing stages, whereas Mayer *et al.* (1993) were unable to detect $E_2$ in gravid female *N. ophidion*. Both

Table 1.1. Descriptive studies of plasma hormone levels, endocrine glands, and target tissues in syngnathids.

| Species | Evaluated[1] | Major Findings[1] | Reference |
|---|---|---|---|
| *H. guttulatus* and *H. hippocampus* | Pituitary histology in males | • Corticotropes: seasonal cycle with most activation at breeding and during pregnancy. Correlated with activity of pouch connective tissue.<br>• Lactotropes: regression during gonadal development, rapid activation at breeding, regression during and after pregnancy. Close correspondence with pouch secretory epithelium. | Boisseau 1967a, b |
| | Interstitial (Leydig) cell histology in males | • Maximum activity prior to mating, regression during and after pregnancy.<br>• Interstitial cell activation accompanies pouch differentiation in juveniles and seasonal pouch development in prebreeding adults. | |
| | Interrenal histology in males | • Interrenal inactive in winter, activation accompanies seasonal pouch development, maximal activity at beginning of pregnancy, decrease afterward.<br>• Parallels corticotrope cycle. | |
| *N. ophidion, S. typhle, S. acus* | Plasma T, KT, OHT, KA, OHA, 17,20-P, $E_2$ by radioimmunoassay in both sexes | • OHA predominant androgen in males and females<br>• Androgens decline in males from mating to pregnancy<br>• $E_2$ present in breeding males and gravid females<br>• 17,20-P nondetectable in breeding and brooding males, gravid females. | Mayer *et al.*, 1993 |
| *H. abdominalis* | T, $E_2$ by radioimmunoassay in females | • T highest in previtellogenic females<br>• No changes in $E_2$ associated with vitellogenesis or ovarian development | Poortenaar *et al.*, 2004 |
| *S. fuscus* and *S. floridae* | Whole brain AVT content in pregnant males | • AVT elevated during mid-gestation | Ripley & Foran 2009a |

[1]Abbreviations: T: testosterone; KT: 11-ketotestosterone; OHT: 11β-hydroxytestosterone; KA: 11-ketoandrostenedione;
OHA: 11β-hydroxyandrostenedione; 17,20-P: 17α,20β-dihydroxy-4-pregnen-3-one; $E_2$: estradiol; AVT: arginine vasotocin

studies propose potential explanations for the variable androgens and failure to detect estrogen, including relatively infrequent sampling, pooled samples, assay sensitivity, and capture-induced stress. Mayer *et al.* (1993) were also unable to detect 17,20-P in their wild samples, although Begovac & Wallace (1988) found it to be the most effective among several progestins in inducing cultured oocytes to undergo final maturation in the Gulf pipefish, *S. scovelli* (Evermann & Kendall). Whereas these studies suggest that circulating steroid hormone measurement is feasible in syngnathids, they again underscore the need for more comprehensive seasonal studies to help identify the predominant endogenous steroids and better understand their secretory patterns. If such studies are well integrated with an understanding of underlying gonadal development, they may provide new insight on fundamental questions of fish reproductive endocrinology. For example, depending on the species, syngnathids have either an asynchronous or a group-synchronous type of ovary where oocytes are produced in sequential cohorts at progressive stages of maturity (Begovac and Wallace, 1987; Selman et al., 1991; Sogabe et al., 2008). It would be of interest to see whether syngnathids with asynchronous vs. group-synchronous oogenesis exhibit distinct hormonal profiles during the reproductive cycle.

*Brood pouch development*

With a function analogous to the mammalian uterus, the syngnathid brood pouch provides a unique opportunity for the examination of the comparative endocrinology of

pregnancy (Stölting and Wilson, 2007). Several experimental approaches have been utilized to elucidate the hormonal regulation of brood pouch development and function. Table 1.2 summarizes experimental endocrine studies undertaken in syngnathids, many of which have focused on brood pouch function. Several of these studies suggest that the development of the brood pouch prior to pregnancy is dependent on androgens that are likely of testicular origin. When Boisseau (1967a) castrated non-pregnant male *H. hippocampus* and *H. guttulatus* seahorses that had a fully developed brood pouch, hypoplasia of the connective tissue and a rapid decrease in the blood supply to the brood pouch were observed. These effects were reversed with prolonged injections of testosterone propionate (TP) following castration. Likewise, post-breeding intact males were able to reconstitute the internal structure of the brood pouch in response to exogenous T administration. Hypophysectomy (removal of the pituitary) resulted in involution of Leydig cells (as would be expected if testicular steroid secretion is under gonadotropin control) accompanied by hypoplasia of the epithelium and connective tissue of the brood pouch (Boisseau, 1967a). Injections of TP following hypophysectomy restored the internal structures of the brood pouch, which suggests that androgens mediate the growth and maintenance of the brood pouch in non-pregnant males. Interestingly, both Boisseau (1967a) and Noumura (1959) observed that testosterone administration to females elicited development of a rudimentary brood pouch. Once the predominant endogenous androgens have been identified in syngnathids, it will be necessary to replicate these studies with physiological doses to

Table 1.2. Experimental endocrine studies in syngnathids.

| Species | Evaluated | Experimental manipulation[1] | Major Findings[1] | Reference |
|---|---|---|---|---|
| *S. schlegeli* | Brood pouch histology | Castration of non-pregnant males | • No regression of brood pouch structures 40 days post-castration. | Noumura, 1959 |
| | | Ovariectomy + T in females | • T stimulates incomplete brood pouch formation in both intact and ovariectomized females. | |
| *H. guttulatus* and *H. hippocampus* | Brood pouch histology, progress of pregnancy | Intact, pregnant males + T, $E_2$, P, ACTH, PRL | • T stimulates pouch development in postbreeding males.<br>• T and $E_2$ disrupt normal pregnancy.<br>• PRL and ACTH stimulate pouch tissues early in pregnancy. | Boisseau, 1967a, b |
| | | Castration + T, $E_2$, P, cortisone, ACTH, PRL in pregnant males | • Castration has no effect on progress of pregnancy.<br>• T, $E_2$ disrupt pregnancy and embryonic development in castrates. | |
| | | Hypophysectomy + T, $E_2$, P, cortisone, ACTH, PRL in prebreeding and pregnant males | • Hypophysectomy and castration cause pouch regression, reversed by T, PRL, ACTH, glucocorticoids in prebreeding males.<br>• Hypophysectomy early in pregnancy causes interrenal regression, pouch regression, embryonic abnormalities, abnormal births in pregnant males.<br>• ACTH, cortisone, and P reverse pregnancy-disrupting effects of hypophysectomy in pregnant males. | |
| | Ovarian and pouch histology | Hypophysectomy, ovariectomy + T, $E_2$, P, gonadotropin, ACTH, glucocorticoids in females | • Hypophysectomy causes ovarian regression.<br>• Ovariectomy and all hormones stimulate rudimentary pouch-like structure. | |
| *H. guttulatus*, *H. hippocampus* and *H. kuda* | Behaviour | Injection of IT and OT in non-pregnant males | • IT and OT induce the complete suite of parturition movements in non-pregnant males | Fiedler, 1970 |

Table 1.2. Continued.

| Species | Evaluated | Experimental manipulation[1] | Major Findings[1] | Reference |
|---|---|---|---|---|
| *S. scovelli* | Germinal vesicle breakdown (GVBD) | *In vitro* ovarian follicle culture with 17,20-P | • 17,20-P induced 100% GVBD in largest follicles, 20-80% in smaller follicles | Begovac & Wallace, 1987 |
| *S. fuscus* and *S. floridae* | Whole brain AVT content | PCB Aroclor 1254 exposure of pregnant males | • AVT content increased by the PCB Aroclor 1254 | Ripley & Foran, 2010 |
| *H. abdominalis* | Plasma cortisol | Confinement, transport stress in both sexes | • Cortisol elevations sustained up to 2 hours after chronic stress, no response to acute stress | Wright *et al*, 2007 |
| *S. scovelli* | Liver and gonad histology, plasma vitellogenin, ornamentation | Ethinylestradiol ($EE_2$) exposure of non-pregnant males | • $EE_2$ Increased liver and testis mass, vitellogenin production, female ornamentation, disruption of spermatogenesis | Ueda et al., 2005; Partridge *et al.* 2010 |

[1]Abbreviations: T: testosterone; 17,20-P: 17α,20β-dihydroxy-4-pregnen-3-one; $E_2$: estradiol; P: progesterone; ACTH: (porcine) adrenocorticotropic hormone; PRL: (ovine) prolactin; IT: isotocin; OT: oxytocin; PCB: polychlorinated biphenyl; AVT: arginine vasotocin; $EE_2$: Ethinylestradiol

hypothalamo-pituitary-testicular axis.

*Male pregnancy*

Although androgens likely mediate brood pouch development in non-pregnant males, they do not appear to be necessary during pregnancy and are likely detrimental during this period. After observing Leydig cell regression during pregnancy, Boisseau (1967b) found not only that pregnancy continued normally in castrated male *H. hippocampus* and *H. guttulatus* seahorses, but that injections of TP during the first half of pregnancy resulted in premature parturition and embryonic deformities in both intact and castrated male seahorses (Boisseau, 1965). Diminished androgens may thus be necessary for pregnancy to proceed normally. Pregnant male *S. typhle* had lower levels of plasma androgens (T; 11-ketotestosterone, KT; and hydroxytestosterone, OHT) than did non-pregnant males in breeding condition (Mayer *et al.*, 1993). Several other species of teleosts have been shown to exhibit decreasing androgens when transitioning between mating behaviour and paternal care (Borg, 1994; Knapp et al., 1999; Mayer et al., 2004; Oliveira et al., 2002). Some of the costs of elevated androgens include direct or indirect energetic costs, immunosupression, predation, wounding, and conflict with pair formation or parental care (Dijkstra et al., 2007; Kurtz et al., 2007; Wingfield et al., 1997). Thus, a reduction in androgens following mating may be an essential trade-off that maximizes fecundity or survival. Diminished androgen production may be particularly crucial for male syngnathids. It may serve to protect developing embryos from masculinization while also serving to ensure that a synchronized cohort of

spermatids is available for subsequent rapid activation to spermatozoa for fertilization immediately after parturition.

Pituitary hormones appear to be important regulators of brood pouch development, pregnancy, and parturition in seahorses, either through direct effects on brood pouch tissues or through tropic effects on peripheral endocrine glands. Boisseau (1967b) observed that hypophysectomy in *H. hippocampus* and *H. guttulatus* seahorses resulted in hypoplasia of the brood pouch as well as disruptions of pregnancy, with premature births and embryonic abnormalities being most common when the procedure was performed during the first half of pregnancy. Histological changes in lactotrophs (pituitary cells secreting prolactin, PRL) paralleled development of the secretory epithelium of the brood pouch (Table 1.1). Corticotrophs (pituitary cells secreting adrenocorticotropic hormone, ACTH) became intensely activated immediately before and during pregnancy, closely corresponding to changes in interrenal activity. These studies implicated both PRL and ACTH (most likely acting through stimulation of glucocorticoid secretion from the interrenal) in driving development and maintenance of the brood pouch. Boisseau (1967b) subsequently found that injections of ACTH or glucocorticoids were capable of stimulating pouch growth and reversing hypophysectomy-induced pouch hypoplasia and disrupted pregnancy. Similarly, PRL powerfully stimulated the brood pouch epithelium in intact and hypophysectomized *H. hippocampus* and *H. guttulatus* seahorses (Boisseau, 1969). More recently, immunoreactive PRL has been detected in pouch fluid of Barbour's seahorse, *H. barbouri* Jordan and Richardson, using a heterologous radioimmunoassay (Patron *et al.*,

2008). Interestingly, progesterone was the only reproductive steroid tested capable of preventing pouch regression and premature birth following hypophysectomy, although this effect may have been achieved through a mechanism that mimics glucocorticoid actions (Boisseau, 1967a). Boisseau concluded that glucocorticoids and PRL function synergistically to maintain the brood pouch in a condition capable of supporting embryonic development (Boisseau, 1967a). However, it should be noted that ACTH and PRL actions have only been examined in a single seahorse genus, employing relatively impure, heterologous hormones. Examination of the biological activity of homologous hormones, particularly PRL, in additional syngnathid species is needed to confirm their broader significance in regulating brood pouch function.

There is a growing body of evidence that the brood pouch epithelium functions in osmoregulation of the pouch fluid (Partridge et al., 2007; Ripley, 2009; Ripley and Foran, 2009a; Stölting and Wilson, 2007). This function has been supported by the identification of mitochondria-rich cells (MRCs, also known as chloride cells) in the brood pouch of several species of *Syngnathus* pipefish (Carcupino et al., 1997, 2002; Serkov et al., 2007; Watanabe et al., 1999). These MRCs contain the ion transport protein $Na^+$,$K^+$-ATPase and have a high degree of structural similarity to those in the gill epithelium (Serkov et al., 2007; Watanabe et al., 1999). The synergistic actions of glucocorticoids and PRL on pouch function is reminiscent of the established roles of these two hormones in mediating permeability and ion transport across gill and gastrointestinal epithelia during freshwater to saltwater transitions in euryhaline teleost fishes (Sakamoto and Mccormick, 2006). Glucocorticoids and PRL also have synergistic

actions in activating secretion in the lactating mammary gland (Ben-Jonathan *et al.*, 2006). Future studies of the endocrine control of the osmoregulatory function of the brood pouch during pregnancy may thus help identify broadly conserved cellular mechanisms through which PRL and corticosteroids interact to regulate salt and water transport across secretory epithelia.

Much less is known about the function of other pituitary hormones in syngnathids. The gonadotropins and thyrotropin have not been investigated, and only one study has attempted to measure growth hormone (Patron *et al.*, 2008). Similarly, little is known about the role of the neuropeptides isotocin (IT) and AVT in syngnathids, but data from other teleosts suggest they are likely mediators of reproductive physiology and behavior. Isotocin is the fish ortholog of the mammalian hormone oxytocin (OT). OT induces uterine contractions during labor in mammals. It also activates the milk let-down reflex and is involved in sexual behavior, pair bonding, and maternal behavior (Nelson, 2000). Much less is known about the role of IT in fish physiology and behavior. Several studies in fishes have shown that injections of IT induce the spawning reflex in both males and females (Goncalves and Oliveira, 2011). In syngnathids, IT may be involved in inducing parturition at the end of pregnancy. Injections of IT and OT into the wall of the brood pouch of non-pregnant *H. hippocampus*, *H. guttulatus*, and the spotted seahorse, *H. kuda* Bleeker, resulted in the complete sequence of behaviours observed during the normal birthing of fry (Fiedler, 1970). Contrary to data from other teleosts, IT and OT did not induce spawning behavior in males. It should be noted that no experiments have been conducted with IT/OT in female syngnathids or with AVT (the ortholog of the

mammalian hormone arginine vasopressin, AVP) in either sex. AVT was more effective than OT in inducing parturition in female guppies, *Poecilia reticulata* Peters (Kujala, 1978). AVT also has established roles in osmoregulation, metabolism, vasomotor responses, reproductive behavior, and the stress response in fishes (Balment *et al.*, 2006). Considering the functions of AVT overlap with those proposed for the functions of the brood pouch (Stölting and Wilson, 2007), it is a likely candidate for mediating diverse aspects of male pregnancy in syngnathids, including brood pouch or testicular myoid cell contraction, pouch fluid osmotic control, or affiliative behaviour. Male dusky pipefish, *S. floridae* (Jordan & Gilbert), and Northern pipefish, *S. fuscus* Storer, showed a 17-fold change in whole-brain AVT concentration (measured with a commercial ELISA) from peak levels during pregnancy to post-parturition levels (Ripley and Foran, 2009a). The ability to measure physiologically significant AVT levels with commercial kits is an exciting development that should provide an opportunity for more extensive exploration of the role of AVT in the endocrine regulation of testis and brood pouch function. The established role of AVT in activating contraction of reproductive ducts during oviposition (Norris, 2007) makes this hormone an intriguing candidate for eliciting the muscular contractions involved in egg and sperm release during mating, as well as parturition.

The experimental studies in seahorses by Boisseau (1967a) and the relatively few recent studies of hormone measurement in pipefish suggest a potential model (Fig. 1.3)

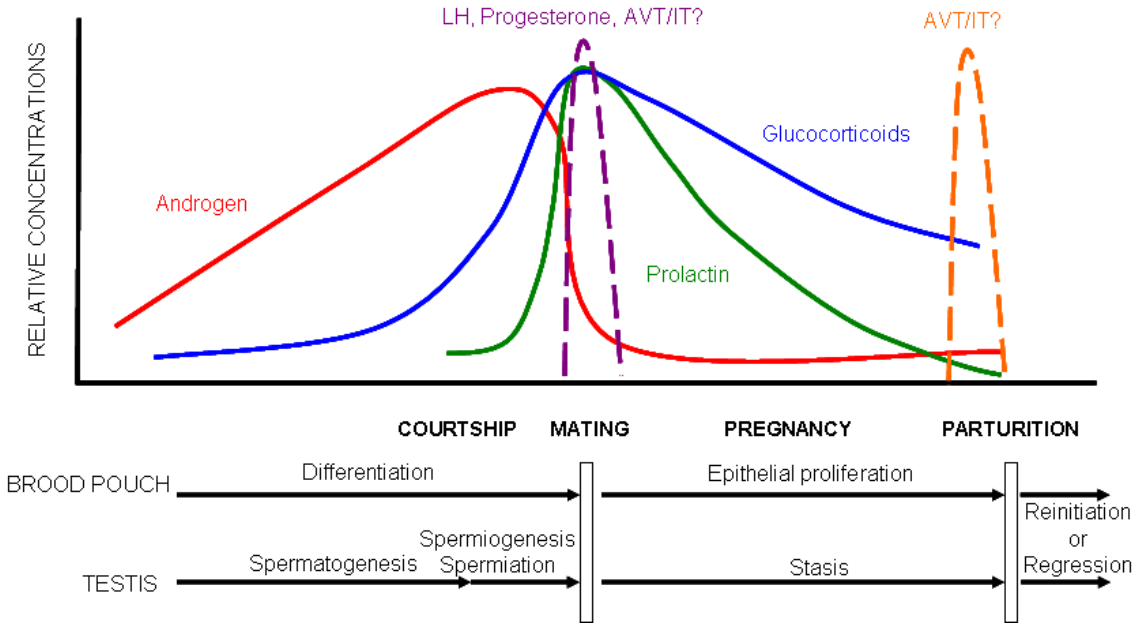



Fig. 1.3. Suggested changes in pituitary and steroid hormones during a single cycle of brood pouch development and pregnancy in a male syngnathid, based primarily on studies in seahorses and established functions in non-syngnathid species. See text for details.

for the endocrine control of spermatogenesis and pregnancy in syngnathids with enclosed brood pouches (Wilson *et al.*, 2001). At the onset of the breeding season, pituitary gonadotropins activate spermatogenesis and concomitant steroidogenesis in the testis that result in the increase of the testicular production of androgens. These androgens serve not only to promote the progression of spermatogenesis toward production of a predominant population of spermatids, but also to stimulate sexual characters, including differentiation of the brood pouch epithelium. Circulating androgen levels increase during the progression of spermatogenesis similar to other teleost fishes (Schulz *et al.*, 2010), but they rapidly decrease around mating and remain low during pregnancy. As in other teleosts, acute elevation of LH and a progestin MIS may accompany mating. At this time, AVT may also function to activate smooth muscle contraction to expel sperm from the muscular testis. After mating, the pouch seals and the synergistic actions of PRL and glucocorticoids promote pouch stromal proliferation and epithelial secretion. These cellular changes may promote alterations in epithelial function that result in a reduction in the osmolarity of the brood pouch fluid (Partridge *et al.*, 2007; Ripley, 2009), possibly through activation of ion transporters in MRCs (Watanabe *et al.*, 1999). As PRL levels decrease during pregnancy, the singular action of glucocorticoids results in apoptosis of the brood pouch epithelial cells (Carcupino et al., 1997, 2002; Sakamoto and Mccormick, 2006). These changes result in an increased permeability of the brood pouch to $Na^+$ and osmolarity of the brood pouch fluid is either actively regulated through $Na^+$ secretion into the brood pouch (Partridge *et al.*, 2007) or passively through selective diffusion of $Na^+$ from the surrounding seawater. The total

osmolarity of the brood pouch fluid increases to near that of seawater prior to fry release at parturition (Partridge *et al.*, 2007; Ripley, 2009). Parturition may be induced by a spike in plasma AVT and/or IT that results in contraction of the smooth muscle of the brood pouch. For syngnathids that have a series of sequential pregnancies, the cycle would begin again with an increase in testicular androgens, spermatogenesis, and a new round of brood pouch epithelial proliferation in preparation for the next pregnancy.

*Reproductive behavior*

Reproductive behaviour in syngnathids generally includes a courtship phase followed by spawning in which the female transfers eggs to the male (Berglund and Rosenqvist, 2003; Vincent, 1994). Even though all male syngnathids have some form of pregnancy (Wilson *et al.*, 2001), some species have conventional sex roles (greater male intrasexual competition) and others are sex-role reversed (greater female intrasexual competition; Vincent *et al.*, 1992). Often sex-role reversed species are characterized by females with secondary sexual characters, a greater variance in female than male mating success, and greater choosiness by males (Eens and Pinxten, 2000). Despite the unique sexual behaviour of syngnathids, there have been no studies specifically addressing hormonal regulation of reproductive behavior. However, measurement of steroid hormones in reproductively active animals has provided some clues as to the endocrine regulation of sex-role reversed behaviors.

When considering sex-role reversed species, researchers originally predicted a reversal of the major sex steroids, T (and/or KT for fish) and $E_2$ (Eens and Pinxten, 2000). However, several studies measuring sex steroids in syngnathids do not support this hypothesis. Mayer *et al.* (1993) found that the steroid hormone profiles of T, KT and $E_2$ in *S. typhle, S. acus,* and *N. ophidion* were similar to those of fish species with conventional sex-roles (Borg, 1994) despite the reversed sex-roles observed in these species (Vincent *et al.*, 1995). In addition, manipulation studies have shown induction of sex-specific traits as would be predicted for species with conventional sex roles (Boisseau, 1967a; Noumura, 1959; Partridge et al., 2010; Ueda et al., 2005). Taken collectively, these studies suggest that syngnathids may be similarly constrained as other vertebrates: the characteristic sex steroid produced by the gonad (e.g. T, KT for males, and $E_2$ for females) mediates reproductive function and secondary sexual characters whereas elevated levels of non-sex-typical steroids result in a decrease in reproductive function.

If the characteristic gonadal steroids in syngnathids mediate sex-typical processes, researchers are still left with the puzzle of how sex-role reversed behavior is mediated. It is possible that less conventional sex steroids are involved. Mayer *et al.* (1993) found females had similar or higher levels of the androgens OHA and/or KA than did males in all three species of pipefish studied. Within females, OHA was usually the sex steroid found in the highest concentration in the plasma and was two fold to eight fold higher than $E_2$. Although OHA is often considered a weak androgen (Lokman et al., 2002b), it is commonly used in aquaculture to induce female-to-male sex reversal in fish (Baron et

al., 2008a; Kuzminski and Dobosz, 2010). Whether OHA and KA have significant physiological functions in syngnathids is not known. These high levels of OHA and/or KA may be related to the sexual dimorphism and female aggressive behavior seen in these species. Preliminary evidence suggests that OHA is involved in female competitive aggression in *S. scovelli* (Scobell *et al.*, *In prep*), a highly polyandrous sex-role reversed species (Jones and Avise, 1997). However, further studies of the effects of these steroids on reproductive behavior, coupled with measurement of circulating levels, will be necessary before their significance as physiological regulators of behavior can be established.

Neuropeptides such as AVT, IT, and α-melanocyte stimulating hormone (α-MSH), should also be of interest in studies of syngnathid reproductive behavior. AVT/AVP has been linked to aggressive behaviors in most vertebrate classes (Goodson and Bass, 2001). This neuropeptide is a likely candidate for mediating the aggressive and/or affiliative behaviors involved in sex-role reversal because it can influence social behavior in fish independent of the gonadal steroid hormones (Foran and Bass, 1999; Goodson and Bass, 2000; Semsar and Godwin, 2003). Several studies also suggest that AVT promotes courtship behavior in fish (Goncalves and Oliveira, 2011). There have been no studies in syngnathids that test the relationship of AVT with aggressive or courtship behavior, but a recent study is suggestive that this hormone may be involved in sex-role reversed behavior in syngnathids. Ripley & Foran (2009b) reported that whole-brain AVT content in gravid female *S. floridae* and *S. fuscus* was comparable to that of pregnant males and higher than non-pregnant males. Comparative studies that correlate

AVT neuropeptide levels with behavior using species where males and females differ in their levels of competition and courtship should help elucidate whether this hormone is involved in mediating sex-role reversed behavior. Such studies should be integrated with a more detailed examination of the expression of AVT and IT and their receptors in the CNS to help establish potential behavioral roles during courtship, mating, and pregnancy. Although only a few studies have investigated the role of IT in reproductive behavior in fishes, the results suggest that IT reduces aggressive behavior (Goncalves and Oliveira, 2011). Comparative studies that examine the role of IT in syngnathids may help to elucidate why male syngnathids with reversed sex-roles are less competitive for mates than those with conventional sex-roles (Vincent *et al.*, 1992).

In several sex-role reversed species, the females are ornamented and some also develop a striking nuptial coloration during courtship and/or competitive displays (Berglund, 2000; Bernet et al., 1998; Rosenqvist, 1990; Scobell et al., *In prep*). The physiological mechanism(s) underlying this phenomenon have not been studied in syngnathids, but one potential candidate is α-MSH, a neuropeptide that stimulates female nuptial coloration in two-spotted gobies, *Gobiusculus flavescens* (Sköld *et al.*, 2008). α-MSH is part of the melanocortin system, which may have pleiotropic effects not only on coloration but also on sexual and aggressive behavior as well as resistance to stress (Dijkstra et al., 2009; Ducrest et al., 2008). Thus, α-MSH has the potential to mediate a suite of traits in sex-role reversed syngnathids including female nuptial coloration, courtship, and competition. However, rapid pigmentary responses in many

teleosts are under direct neural control, so non-endocrine mechanisms should also be considered (Norris, 2007).

*Endocrine disruption of reproduction*

Due to their distribution in shallow, coastal habitats (Koldewey and Martin-Smith, 2010) and their unusual mode of reproduction, syngnathids may be particularly susceptible to exposure to anthropogenic endocrine-disrupting chemicals. They may thus serve as an important group of fish for detecting biomarkers of endocrine disruption during the ecological risk assessment process (Hutchinson *et al.*, 2000). Ueda *et al.* (2005) and Partridge *et al.* (2010) showed that exposure to ethinylestradiol ($EE_2$), an estrogenic compound normally found in human wastewater effluent, resulted in the development of female-like secondary sexual characters and disrupted reproduction in male *S. scovelli*. Ripley & Foran (2010) exposed pregnant male *S. floridae* and *S. fuscus* to the polychlorinated biphenyl Aroclor 1254 (an industrial ecotoxicant) and found that both species had elevated levels of AVT in the brain compared to controls. Although the specific functions of this increase in AVT are unknown, an increase in AVT expression has been demonstrated in fishes in response to a variety of stressors (Balment *et al.*, 2006). Cortisol is established in vertebrates as a stress response hormone (Hazon and Balment, 1998) and would also be of interest to measure in studies of endocrine disruption. Cortisol has been shown to be released following transport stress in *H. abdominalis* (Wright *et al.*, 2007), but changes in cortisol levels have not yet been

examined in relationship to endocrine disruption in syngnathids. This would be particularly interesting in the context of pregnancy, given Boisseau's (1967a) conclusion that glucocorticoids play a central role in maintenance of brood pouch function during pregnancy. Further studies are needed to determine which endocrine disruptors pose the greatest threat to particular populations of syngnathids and how exposure affects reproductive physiology and behavior.

**Future directions**

Although syngnathids are a productive taxon in which to study the evolution of reproductive hormone function, much of the fundamental reproductive biology needed to provide a physiological context for interpretation of endocrine studies has yet to be elucidated. The application of modern biochemical and molecular biological techniques (Mobley *et al.*, 2011), along with improved husbandry practices (Koldewey & Martin-Smith, 2010), is beginning to provide researchers with the tools for better understanding hormonal function in the family Syngnathidae. Future endocrine studies must focus on syngnathid species with well-characterized reproductive biology, incorporating both laboratory and field sampling to establish the identity of the principal endogenous hormones and define physiological ranges for experimental manipulations. Sensitive immunoassays for multiple hormones in small volumes, such as high/ultra performance liquid chromatography followed by mass spectrometry (Blasco *et al.*, 2009), should be validated for syngnathid peptide and steroid hormones. Steroid measurement in fish

holding water may also be a practical, non-invasive method for small or rare syngnathids (Scott and Ellis, 2007) or for behavior studies where repeated sampling of one individual is required (Kidd et al., 2010; Scott et al., 2008). Quantitative real time PCR and *in situ* hybridization techniques for assessing steroidogenic enzyme, peptide hormone, and hormone receptor expression in the central nervous system, pituitary, gonad, and brood pouch should be developed as extremely sensitive means to elucidate patterns of secretion and identify potential hormone targets. Coupled with immunocytochemistry for receptor protein expression, these studies should begin to assess changes in target sensitivity throughout reproductive cycles. Finally, hormone administration studies are needed to establish actions of steroid and protein hormones in syngnathid species using established immersion (Partridge et al., 2010; Ueda et al., 2005) or implantation (Mylonas and Zohar, 2001) techniques. Efforts should be made to use recombinant fish hormones when possible. The challenge of extirpation-replacement studies may be partially overcome using hormone receptor agonists and antagonists, as have been productively applied to examine the role of neurohypophysial peptides in the regulation of reproductive smooth muscle contraction (e.g., in mating or parturition) and behavior in other teleosts (Backström and Winberg, 2009; Lema and Nevitt, 2004; Semsar et al., 2001).

Once these sensitive techniques are more broadly available for syngnathids, their exceptional reproductive biology should provide a unique opportunity to address several fundamental questions of hormone function in teleost fish. Among the most intriguing are those that relate to the comparative endocrinology of PRL. Whereas the primary

established role of PRL in fish is osmoregulatory, it also has been shown to regulate skin secretions, paternal behavior, immune function, and growth (Kawauchi et al., 2009; Pall et al., 2004), all proposed functions of the brood pouch function. Studies of syngnathid PRL, while helping elucidate the osmoregulatory function of the pouch, will provide a unique opportunity to assess how hormonal regulation of integumentary function has evolved as brood pouch morphology has diversified (Wilson *et al.*, 2001). Syngnathids also provide a model in which to examine the regulation and fitness consequences of the androgen to PRL transition that occurs from mating to parental behavior, a phenomenon conserved across vertebrate classes (Pall et al., 2004; Pall et al., 2002; Ziegler, 2000). Considering all male syngnathids have some form of brooding, but not all are sex-role reversed, comparative studies of sexual and paternal behavior, androgens, and PRL should help elucidate behavioral functions of these hormones during the reproductive cycle. Other factors such as immunocompetence and protection of embryos from steroid exposure could be examined simultaneously (Kurtz et al., 2007; Mayer et al., 2004). Studies of syngnathid brood pouch function must include glucocorticoids to better understand their role in regulation of brood pouch function and identify conserved aspects of their synergistic action with PRL. Additionally, glucocorticoids should be more broadly included in studies of stress responses and reproductive behavior. Comparative studies of hormonal regulation of behavior in syngnathids should help identify whether novel steroid hormones and neuropeptides have acquired a regulatory function in sex-role reversal. Once these mechanisms are established, syngnathid studies promise to be particularly productive for the evaluation of the effects of endocrine

disruption on fitness, as viability of retained embryos can be directly assessed in the brood pouch. Although such studies may appear challenging, syngnathid endocrinologists should take inspiration from studies of sex-changing reef fish, in which field and laboratory studies have successfully integrated descriptive endocrinology with experimental approaches to provide an emerging synthetic understanding of complex endocrine processes in small teleost fish (Munakata and Kobayashi, 2010).

**Dissertation justification**

Although the studies of reproductive endocrinology in syngnathids suggest there are many intriguing physiological constraints and adaptations in this Family, further studies are needed across a variety of species before we can draw conclusions about how these mechanisms evolved. I chose the highly polyandrous, sex-role reversed pipefish, *Syngnathus scovelli*, to study the role of androgens in male pregnancy and female intrasexual competitive behavior in a syngnathid. *The central hypothesis of this dissertation is that androgens play a central role in the mediation of both male reproductive physiology and female behavior in the family Syngnathidae.* To address this hypothesis, I first tested the predictions of the model put forth in **CHAPTER I** with respect to the role of the androgen 11-ketotestosterone across the reproductive cycle in males. I then examined how injections of KT, as well as OHA and cortisol, affected female intrasexual competitive interactions. Lastly, I determined how injections of KT affected female intrasexual competition in a dose-dependent manner.

# CHAPTER II

## THE ROLE OF 11-KETOTESTOSTERONE IN THE MALE REPRODUCTIVE CYCLE OF *Syngnathus scovelli*

Male pregnancy, sex-role reversal, and the variety of mating systems in the Family Syngnathidae have spurred significant research in the fields of behavioral ecology and molecular genetics (Berglund and Rosenqvist, 2003; Jones and Avise, 2001b; Vincent et al., 1992). Research in these fields has been fruitful and has provided insight into the underlying assumptions of sexual selection theory. However, research that aims to understand the proximate mechanisms underlying these unique phenomena has lagged behind that of other fields. Hormones are well established in teleost fish as primary regulators of puberty, gonadal development, mating, and reproductive behaviors (Lubzens et al., 2010; Munakata and Kobayashi, 2010; Schulz et al., 2010; Zohar et al., 2010). The diversity of reproductive function and behavior throughout the family Syngnathidae makes them an intriguing taxon in which to examine the degree of conservation of endocrine function compared with other teleost fish. However, our understanding of the basic hormonal regulation of gonadal function and reproductive behavior lags well behind that of most established teleost reproductive model species.

Several studies in syngnathids have provided useful preliminary data for understanding some of the most fascinating questions in this field. Do the same hormones mediate male pregnancy in syngnathids as mediate pregnancy in mammals? How do hormones function to regulate alternating cycles of spermatogenesis and

pregnancy in males? Does sex-role reversal depend upon a reversal of reproductive steroid function in males and females? Answers to these questions have profound implications for the ability of natural and sexual selection to shape reproductive physiology and behavior in syngnathids.

Male pregnancy is a phenomenon found only in the Family Syngnathidae. The location and complexity of the brood pouch structure varies among species within the family (Carcupino et al., 2002; Kornienko, 2001; Stölting and Wilson, 2007; Wilson et al., 2003). The brood pouch is located on the ventral tail in the subfamily Urophori and on the ventral trunk in the subfamily Gastrophori (Wilson *et al.*, 2003). The most basic type of brood pouch is an open surface on the ventral side of the male where eggs from the female are attached by a mucous-like substance (Carcupino et al., 2002; Kornienko, 2001). The epithelium only differs from that of the rest of the body surface in that it is more highly vascularized (Carcupino *et al.*, 2002). Within both the Urophori and Gastrophori lineages, brood pouch complexity increases in more derived taxa (Wilson *et al.*, 2003). The most complex types of brood pouches, like those of *Syngnathus* and *Hippocampus* species, are completely isolated from the environment (Kornienko, 2001; Wilson *et al.*, 2003). They have marked vascularization, epithelial differentiation, and smooth muscle tissue (Carcupino *et al.*, 2002). Complex brood pouches also have mitochondria-rich chloride cells, which are similar to cells that perform an osmoregulatory function in gills of several syngnathid species (Carcupino et al., 2002; Partridge et al., 2007; Ripley, 2009; Watanabe et al., 1999).

In theory, sperm competition could occur in males with open brood pouch types. However, parentage data have shown that all syngnathids studied have 100% paternity within their broods regardless of pouch type (Avise et al., 2002; Jones and Avise, 2001a; Jones et al., 1999; Kvarnemo et al., 2000; Mccoy et al., 2001; Wilson, 2006; Wilson and Martin-Smith, 2007). Consequently, there is no difference in testis mass among syngnathids with different pouch types (Kvarnemo and Simmons, 2004). In addition, syngnathids have remarkably small testis size compared with other fish (Kvarnemo and Simmons, 2004). Accordingly, sperm counts are also much lower than those of other fish. Compared to most species of fish that have hundreds of thousands to millions of sperm (Stockley *et al.*, 1996), sperm counts in syngnathids range from 300-10,000 (Kornienko and Drosdov, 1999; Van Look et al., 2007). The lack of sperm competition, small testis size, and low sperm counts of syngnathids suggest that their hormone profiles during spermatogenesis are also likely different in magnitude or duration from that of other teleost species.

Presently, however, there is no information on the potential endocrine correlates of this unusual syngnathid testicular morphology as few studies have described testicular function in syngnathids in sufficient detail to provide a cellular foundation for endocrine studies. Carcupino et al. (1999) noted that all major types of cystic germ cells were present during the reproductive period in the pipefishes *Syngnathus abaster* and *S. acus*, but that aspects of testicular morphology, including an unusual tubular structure and multinucleated symplastic spermatids, distinguished them from those of other teleost species. Carcupino et al. (1999) did not evaluate how testis morphology changed

seasonally or during pregnancy, but did note that the anatomical simplicity of the testis suggests that pipefish may be an excellent experimental model for the investigation of fish reproduction. Boisseau (1967) noted a similar simplistic morphology in *Hippocampus guttulatus* and *H. hippocampus*. The testis of these animals did not appear to comprise spermatogenic cysts or multiple seminiferous tubules as found in other teleost species (Schulz *et al.*, 2010), but instead comprised a single large tubular structure with a muscular wall and a germinal epithelium containing all stages of spermatogenic development that opened into a single central lumen. This arrangement differs dramatically from the accepted cystic spermatogenic process evident in teleosts (Schulz *et al.*, 2010).

In *H. guttulatus* and *H. hippocampus*, these classical interstitial and tubular compartments of the testis underwent seasonal cycles of activity correlated with spawning (Boisseau, 1967a). There was no period of complete testicular regression; germ cells underwent distinct seasonal cycles of proliferation with an approximately six-month period of spermatogonial proliferation followed by equivalent durations of sequential appearance of spermatocytes and spermatids. Spermatids were the most abundant germ cell of the testis, constituting 50-80% of germ cells throughout the year; spermatozoa were only present for a very brief period immediately preceding mating. Several other studies suggest that male syngnathids may only produce mature spermatozoa after courtship with females has been initiated (Kornienko and Drosdov, 1999; Van Look et al., 2007). In *H. guttulatus* and *H. hippocampus*, interstitial tissue underwent a distinct seasonal cycle of activation beginning in December, reaching

maximal activity immediately prior to mating, and then undergoing a rapid involution during pregnancy (Boisseau, 1967). This cycle suggests a prolonged period of steroidogenesis, which is maximal during proliferation of spermatocytes and development of the brood pouch, but terminates prior to appearance of spermatozoa and mating, reaching minimal levels during gestation. Gestation is followed by a second, although smaller peak of steroidogenesis in the month following parturition (Boisseau, 1967a), suggesting that androgen production is suppressed during gestation. These patterns also suggest that maturation inducing steroids such as progestins, which have been found to promote sperm motility (Schulz et al., 2010), may only be present in the circulation for very brief periods immediately preceding spawning. However, no studies have examined seasonal changes in sex steroid secretion or have even identified the primary steroids produced by the syngnathid testis during spermatogenesis or spawning. These few studies have suggested unique aspects of germ cell and testis morphology that warrant more detailed study to establish their presence across syngnathid species and their potential paracrine or endocrine regulation.

Along with their role in mediating spermatogenesis, androgens likely mediate brood pouch development in non-pregnant males (reviewed in Scobell and Mackenzie, 2011); however, they do not appear to be necessary during pregnancy and are likely detrimental during this period. Boisseau (1964) castrated 31 early to mid-pregnancy male *H. guttulatus* and *H. hippocampus* and found that pregnancy continued normally in all males; males gave birth at the end of the normal term (21 days at 20° C); and the fry that were born were not different from controls. This suggested that the testes were not

necessary for maintaining the pregnancy or for inducing parturition. It was also noted that during pregnancy in control males, the interstitial tissue of the testis involuted. This regression of the testes and the concomitant decrease in androgens from them may be necessary for pregnancy to proceed normally in syngnathids. Pregnant male *S. typhle* had lower plasma levels of T, 11-ketotestosterone (KT), and hydroxytestosterone (OHT) than did non-pregnant males in breeding condition (Mayer et al. 1993); the androgen profile is similar for *S. acus*, though the sample size of breeding males is too small to determine whether this is a species-wide phenomenon. Several other species of teleosts have been shown to have a decrease in androgens when transitioning between mating behavior and paternal care (Borg, 1994; Knapp *et al.*, 1999; Oliveira *et al.*, 2002). This trade-off may be even more crucial for male seahorses and pipefish with such elaborate parental care. Injections of testosterone proprionate during pregnancy resulted in premature parturitions of offspring in both normal and castrated males (Boisseau, 1965). Of the remaining fry that were born at the end of the pregnancy, some showed deformations and developmental delays. However, early to mid-pregnancy may represent a sensitive period, because injections made after day 11 in normal males and day 16 in castrated males produced no considerable effects on the pregnancy or offspring. The decreased androgen production observed during pregnancy in the few syngnathids examined so far may serve to protect developing embryos from masculinization while also serving to assure that a synchronized cohort of spermatids is available for subsequent rapid activation to spermatozoa for fertilization immediately after parturition.

Previous work on syngnathids has suggested an important role of androgens in development of the brood pouch, maintenance of the testes, and spermatogenesis. However, to our knowledge there have not been any studies that correlate circulating plasma androgens with these traits across the reproductive cycle. We conducted a field study of circulating 11-ketotestosterone levels in male and female Gulf pipefish, *Syngnathus scovelli*. We first asked whether there was a reversal of plasma KT levels in the sexes of this polyandrous, sex-role reversed species. We then examined the relationship between KT and gonad mass and body measurements in both sexes. Finally, we compared testis mass and circulating KT levels across various stages of the male reproductive cycle.

## Methods

### *Blood collection in field-caught males and females*

Sexually mature male (n = 11) and female *S. scovelli* (n = 24) were collected from Corpus Christi Bay, Texas, on June 2, 2011. Pipefish were collected by seine (1 x 2 $m^2$ with 2$mm^2$ nylon mesh) from seagrass beds. Immediately following capture, each fish was lightly anesthetized in a 0.06% clove oil solution for 1 minute and a blood sample (around 20 - 30 µl) was taken from the caudal vein via a heparinized syringe fitted with a 26-gauge needle within 6 minutes of capture. Fish were then transferred to a terminal dose of MS-222 (1 mg/ml artificial saltwater; Research Organics #1347A). The blood

sample was transferred to a 0.5 ml microcentrifuge tube and placed on ice. Following all field collection, the blood was centrifuged and plasma was drawn off the top, placed in a new 0.5 ml microcentrifuge tube, and frozen until enzyme immunoassay (EIA) analysis. Fish carcasses were transported on ice to our laboratory at Texas A&M University. They were measured for total length (TL), snout-vent length (SVL), body depth just anterior to the dorsal fin (standard depth, $Depth_{std}$) and at the deepest part of the trunk (maximum depth, $Depth_{max}$), and total mass ($Mass_T$), and gonads were dissected and weighed (wet mass, $Mass_G$). Male reproductive state was classified according to the developmental stage of the embryos: early pregnancy (days 1-5: orange eggs visible through the pouch), mid-pregnancy (days 6-9: visible pigmented eyes on embryos), late pregnancy (days 10-14: pigmented embryos with little to no yolk sac), and non-pregnant (post-partum reproductive male).

*Hormone assays*

Ketotestosterone levels were measured with a commercial EIA kit (Cayman Chemical #582751). The manufacturer's instructions were modified slightly to accommodate the small plasma volume of *S. scovelli* (median 10 µl, range 2-20 µl). Most fish provided enough blood to obtain a 10 µl of plasma sample from an individual fish (n = 14 females and n = 6 males); however, four males had < 10 µl of plasma. Each sample was diluted with phosphate buffered saline (PBS) up to 500 µl total volume. All sample solutions were extracted four times with 2 ml ethyl acetate/hexane (50:50). The

organic phase was decanted into a clean borosilicate test tube following freezing in a ¯80 ºC freezer for 5 - 10 minutes. Samples were dried down under nitrogen in a hot water bath and resuspended with EIA buffer for a 1:55 dilution for females and a 1:60 dilution for males. Samples and standard curve were run in duplicate, and the plate was incubated at 4 ºC overnight to increase the sensitivity of the assay. The next day, the plate was washed, developed, and read at 405 nm on a Vmax Kinetic ELISA Microplate Reader (Molecular Devices, www.moleculardevices.com) according to the manufacturer's instructions. Plasma from the remaining females (n = 10) was pooled and used alongside a pool of previously collected male plasma (collected on April 28, 2008) for assay validation. The average recovery from spiked PBS and male and female plasma pools was 92.8%. The sensitivity of the assay was 1.3 pg/ml (manufacturer's data) and the intra- and inter-assay coefficients of variation were 11% (manufacturer's data) and 10.4%, respectively. Cross reactivity was 2.9% for 11-ketoandrostendione, <0.01% for testosterone (manufacturer's data, respectively) and 1.8% for 11β-hydroxyandrostenedione.

*Statistical analyses*

Male and female plasma KT levels from animals in the field were $\log_{10}$ transformed for normality and were compared with a Student's t test. The effect of each morphological measurement on plasma KT levels was analyzed with linear regression, and the effect of gonad mass on plasma KT levels was analyzed with a second-order

polynomial regression. Although small sample size (n = 2 or 3 in each state) precluded meaningful data analysis, trends were observed in male plasma KT levels and testis mass with respect to reproductive state. One male that was giving birth at the time of sampling was not included in these observations due to the sample size of one and the special nature of this physiological state.

**Results**

Plasma KT levels were 30-fold higher in male than female field-sampled *S. scovelli* (mean ± S.E.M., males: 4147.9 ± 1280.5 pg/ml, females: 133.4 ± 7.6 pg/ml; Student's t test: $t_{22} = 9.85$, $p < 0.001$; Fig. 2.1). Neither female nor male size (TL, SVL, $Depth_{std}$, $Depth_{max}$, $Mass_T$) affected plasma KT levels. There was also no relationship between ovary mass and plasma KT levels in females, but males with larger testes had higher circulating KT levels ($R^2 = 0.71$, $p = 0.01$; Fig. 2.2). Testis mass and plasma KT levels showed similar profiles across the reproductive cycle in males (Fig.2.3). Testis mass was lowest in non-pregnant males and increased to average levels during early and mid-pregnancy. The greatest testis mass was observed during late pregnancy. KT levels were lowest during early and mid-pregnancy when embryos were in the early stages of development. The highest plasma KT levels were observed during late pregnancy. KT levels dropped to mid-range levels in non-pregnant males.

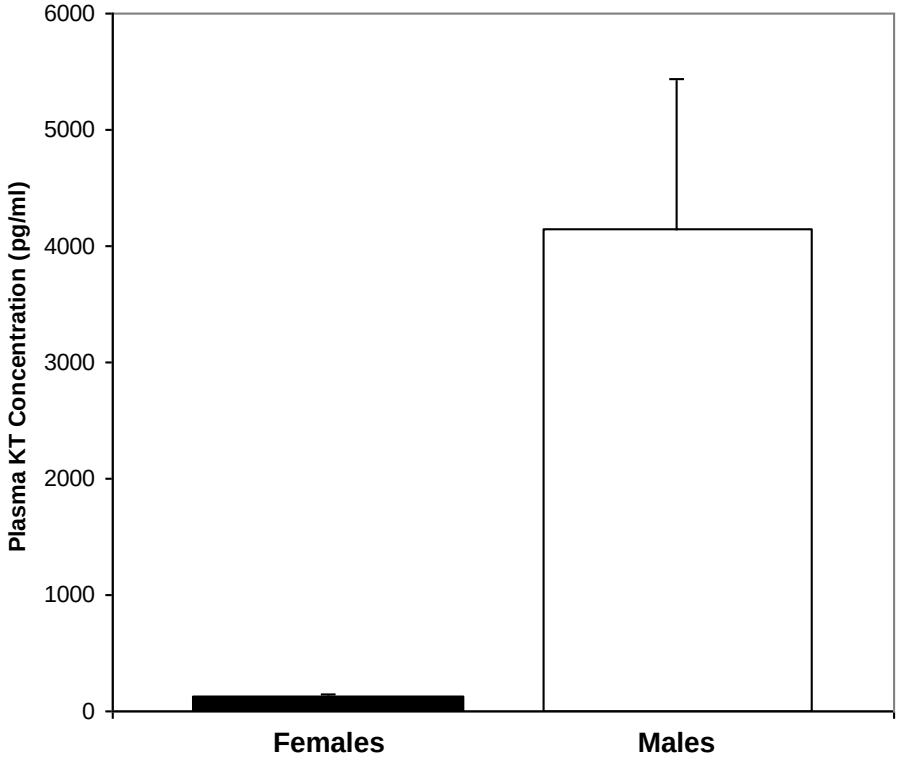



Fig. 2.1. Mean plasma concentrations of 11-ketotestosterone from field-caught male and female *S. scovelli* show the typical teleost hormone profile in this sex-role reversed species. Error bars represent S.E.M.

**Discussion**

Ketotestosterone is the primary androgen that mediates spermatogenesis in fish (Knapp and Carlisle, 2011a), and both KT and testosterone gradually increase over the course of spermatogenesis (Schulz *et al.*, 2010). Spermatogenic males typically have higher plasma KT than females of their species, and plasma KT levels of spawning males can be orders of magnitude higher than females (Lokman et al., 2002b). Despite the male pregnancy and sex-role reversal in *S. scovelli*, plasma KT levels in field-caught males and females showed the typical teleost pattern (Borg, 1994; Lokman et al., 2002b) in that males had substantially higher circulating KT than did females. In addition, KT increased with greater testis mass in *S. scovelli* males in a manner similar to that of androgens in other male vertebrates. The current study supports the data from Mayer et al. (1993) showing no reversal of KT in male and female *S. acus* or *S. typhle* (a polygynanadrous, sex-role reversed species; Berglund *et al.*, 1986; Jones *et al.*, 1999). More field studies of circulating KT in a variety of syngnathids are needed before there is a consensus, but currently there does not appear to be a reversal of the major fish androgen, KT, in sex-role reversed syngnathids.

Several other studies suggest that there is no reversal of the major sex steroids in syngnathids. Female *S. schlegeli* exposed to testosterone developed a crude brood pouch that was less developed yet still similar to that of males (Noumura, 1959). In unaltered females, the ovary that was in direct contact with the testosterone pellet was smaller than the other ovary, had a reduced ovarian cavity, a thickened ovarian wall, and few mature

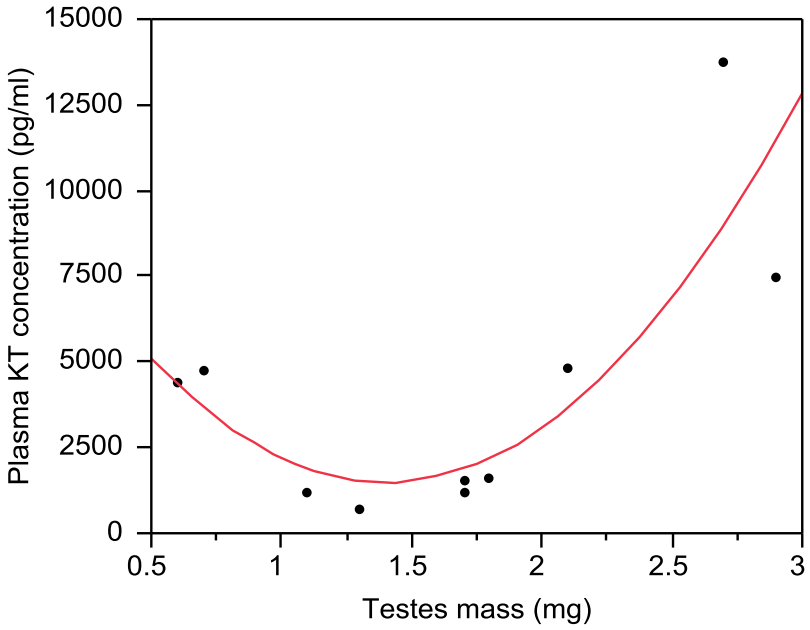



Fig. 2.2. Similar to other male teleost fish, plasma KT concentration increases with greater testis mass despite male pregnancy, the sex-role reversed mating system, and the comparatively small size of testes in *S. scovelli*.

eggs. Conversely, *S. scovelli* males exposed to synthetic 17α-ethinylestradiol ($EE_2$) developed female-like secondary sexual traits after only 10 days (Partridge et al., 2010; Ueda et al., 2005). Exposed males developed iridescent lateral stripes, a trait that is normally found only in sexually mature females. This trait persisted in exposed males up to 3 weeks following transfer to clean water (males were sacrificed at the end of this period). Female-typical hormones also affect male pregnancy adversely. Injections of estradiol benzoate during early pregnancy in *H. guttulatus* and *H. hippocampus* resulted in premature parturitions of offspring in both normal and castrated males (Boisseau, 1965). Injections of progesterone prior to day 12 of pregnancy resulted in premature parturition and developmental abnormalities in castrated males similar to those obtained with estradiol benzoate and testosterone proprionate. Pregneninolone, a synthetic progestin, resulted in the most severe disruption of pregnancy in *H. guttulatus* and *H. hippocampus*. Pregneninolone disrupted pregnancy at all stages in both normal and castrated males. Most pregnant males that were kept in seawater containing 5 mg/L pregneninolone expelled all embryos prior to the end of the term (day 21). Premature parturitions began three or four days after exposure to pregneninolone, and expelled offspring were abnormally developed. Taken collectively, these studies suggest that seahorses and pipefish may be similarly constrained as other vertebrates in that gametogenesis and secondary sexual traits are mediated primarily by the main sex steroid produced by the gonad and high levels of non-sex-typical steroids result in a decrease in reproductive function.

Similar to the effects of female-typical sex steroids, elevated androgens also appear to be detrimental to the initial stages of male pregnancy in syngnathids. Boisseau (1965) showed that androgen injections in early to mid-pregnancy resulted in premature parturition and fry that were deformed in *H. guttulatus* and *H. hippocampus*. In male *S. scovelli* in our study, both testis mass and plasma KT were low during the early and middle stages of pregnancy. The studies by Boisseau (1965, 1967a) suggest that this decrease in testis mass is likely due to involution of the Leydig cells (interstitial tissue). Teleost Leydig cells are stimulated by pituitary luteinizing hormone to produce KT (Miura *et al.*, 1991; Schulz *et al.*, 2010). Thus, involution of Leydig cells in *S. scovelli* would be expected to result in a concomitant decrease decline in circulating KT levels. We did observe this relationship between testis mass and plasma KT in the early and middle stages of pregnancy. If androgens are detrimental to normal embryo development, involution of the testes during early to mid-pregnancy might protect embryos from excessive steroid exposure during critical periods. Alternatively, maintaining elevated levels of androgens (or other sex steroids) during early pregnancy may be a mechanism by which males can affect post-copulatory sexual selection in broods of undesirable mates. The developmental delays and deformations in offspring that Boisseau (1967b) described for *H. guttulatus* and *H. hippocampus* are similar to those observed for male *S. scovelli* showing brood reduction (Paczolt and Jones, 2010). Further studies on the effects of androgens during early pregnancy, their relationship to testis function, and the ultimate success of the brood in syngnathids should not only help

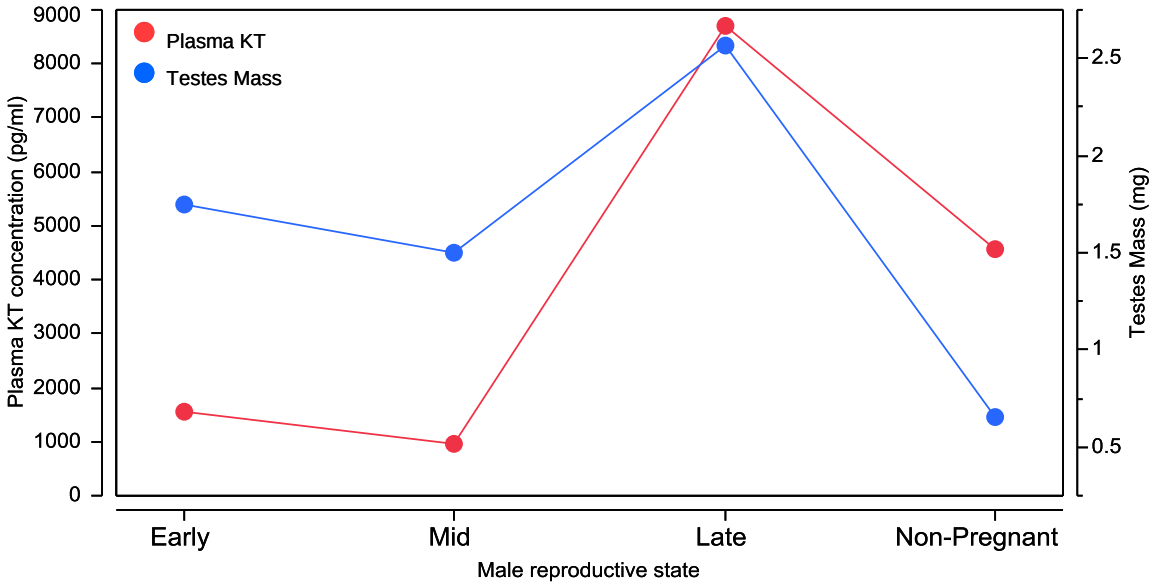



Fig. 2.3. Plasma KT concentration and testis mass in *Syngnathus scovelli* show a similar pattern across the male reproductive cycle. Testis mass and KT levels are low during early and mid-pregnancy, but increase toward the late stage of pregnancy when the male prepares to give birth and get ready for the next spawning event. Both KT and testis mass decrease following birth when the male is in the non-pregnant, breeding state.

us to understand how males transition from breeding to parental care behavior but may also help us to understand post-copulatory sexual selection in this lineage better.

After this apparently sensitive period of embryo development, it seems that high plasma androgens in late pregnancy do not adversely affect the fry. Boisseau (1965) found that androgen injections at the end of the pregnancy had little affect on the continuation of the pregnancy or offspring development. Our data for *S. scovelli* are in accord with this study: circulating KT levels and gonad mass were at their highest in late-stage pregnant males. Because there is no sperm competition in this species (Jones et al., 2001b) and it is likely few sperm are needed to fertilize eggs within the pouch (Kornienko and Drosdov, 1999; Van Look et al., 2007), it appears that males may produce the sperm needed for the next spawning event late in the pregnancy when embryos are insensitive to increased levels of androgens (Boisseau, 1965). It is possible that following this peak of androgens, a maturation-inducing steroid increases in the plasma and activates sperm motility. It will be interesting to see whether spermatids are the predominant germ cell within the testis for most of the breeding cycle and spermatozoa are only produced immediately prior to mating in *S. scovelli* as has been implicated in studies in other syngnathids (Boisseau, 1967a; Kornienko and Drosdov, 1999; Van Look et al., 2007).

The data from our study of *S. scovelli* suggest that there are internal or external cues that help coordinate the regulation of the testes with that of the brood pouch across the reproductive cycle. Following birth when males are once again ready to accept eggs from females and begin a new pregnancy, both testis mass and plasma KT levels in *S.*

*scovelli* decreased. Interestingly, the smallest testis mass that was observed in non-pregnant males resulted in average plasma KT levels. These two males, which largely drive the polynomial relationship in Fig. 2.2, were both relatively small males (TL, $Mass_T$). It is possible these males were recrudescent males that had not yet had their first pregnancy of the season. In this case, elevated KT levels may be activating the development of the brood pouch in preparation for the breeding season as was seen in *H. guttulatus* and *H. hippocampus* by Boisseau (1967a). Alternatively, if these males were between pregnancies, KT might have been produced by extra-testicular sites such as the head, kidney, liver, or blood cells (Borg, 1994). A more extensive examination of the relationship of circulating KT levels with reproductive state, testis mass, and stage of spermatogenesis is necessary to elucidate the role of this androgen in the regulation of reproduction in *S. scovelli*.

Male *S. scovelli* are a good model species for studying the effects of androgens on spermatogenesis and male pregnancy. The duration of gestation in *S. scovelli* is 14-15 days (Scobell *et al.*, 2009). In the laboratory, males usually get pregnant the day after giving birth (Scobell *et al.*, 2009). *S. scovelli* is a year-round breeder, so recrudescent males could potentially have 24-26 pregnancies per year. Embryo development can be viewed through the transparent skin flaps of the brood pouch allowing the fate of individual embryos to be tracked across the pregnancy (Paczolt and Jones, 2010). The use of non-invasive water sampling of KT and other steroids (Kidd *et al.*, 2010) would permit repeated measurement of hormones throughout the pregnancy or over multiple pregnancies. After the role of androgens are established in *S. scovelli*, it would be

informative to do comparative studies of the regulation of the reproductive cycle in species of syngnathids that have different brood pouch complexities and mating systems. Ultimately, we should strive to determine how male reproductive physiology coevolved with the brood pouch and male pregnancy in Syngnathidae.

# CHAPTER III

## HORMONE MANIPULATION OF FEMALE INTRASEXUAL COMPETITIVE AGGRESSION IN THE SEX-ROLE REVERSED GULF PIPEFISH, *Syngnathus scovelli*

The majority of animal mating systems are conventional mating systems, with males competing for access to choosy females. However, a limited number of species are sex-role reversed (Clutton-Brock and Vincent, 1991; Eens and Pinxten, 2000); females compete for access to mates and males are choosy (Vincent *et al.*, 1992). Despite the fact that sex-role reversal is a curious exception to the rule in mating systems, there has been little study of the evolution of sex-role reversal at the physiological level (Eens and Pinxten, 2000; Staub and De Beer, 1997).

Sex-role reversed species provide a unique opportunity to test the assumptions of sexual selection theory because for sex-role reversal to be an evolutionarily stable strategy, females that win competitions must also be good mates (Andersson, 1994). Therefore, selection that acts to increase aggressive behavior in females must do so without greatly hindering reproductive function. Herein lies the fundamental physiological problem for the evolution of female intrasexual aggression: How did females evolve to be both aggressive and reproductive concurrently? Ketterson et al. (2009) proposed that selection likely acts on a continuum with respect to the evolution of hormone mediated suites of traits: at one end, selection produces tightly integrated suites of traits mediated by one hormone, and on the other end, selection decouples the

physiological regulation of these traits such that they can adapt independently (Ketterson *et al.*, 2009). Intrasexual competitive behavior in females could be integrated with the major hormones that regulate reproduction, or alternatively, it could be mediated in a modular fashion by one or more hormones that can affect behavior without influencing reproductive function. From a developmental viewpoint, female competitive aggression in sex-role reversed species could be activated by accessing a male-typical ancestral neural network via androgens (Hausberger et al., 1995; Kern and King, 1972; Kobayashi and Nakanishi, 1999; Watson and Kelley, 1992), or through a female-specific neural network that is mediated with estrogens or progestins (Mccarthy, 2008; Yanase and Gorski, 1976). Whether sex-role reversed female sexual behavior is regulated hormonally in an integrated or independent manner via sex-typical or via non-sex-typical neural pathways is not known.

The teleost family Syngnathidae (seahorses, sea dragons, and pipefish) is an exceptional group in which to study the hormonal mediation of female intrasexual competition. Syngnathids exhibit a range of mating systems from monogamy to polyandry (Jones and Avise, 2001a) and several species are sex-role reversed (Berglund and Rosenqvist, 2003; Jones and Avise, 2001b). In these species, females compete to mate with a male who then becomes pregnant (Berglund and Rosenqvist, 2003). Male *Syngnathus scovelli* prefer large over small females (Paczolt and Jones, 2010). In *S. typhle*, males prefer dominant over subordinate females (Berglund and Rosenqvist, 2001a), and choose on the basis of both behavior and a sexually dimorphic ornament (Berglund and Rosenqvist, 2001a, b). The presence of dominant females can suppress

ornament display and reproductive effort in *S. typhle* (Berglund, 1991; Bernet *et al.*, 1998), and the expression of sexual ornaments in subordinate female *Nerophis ophidion* (Rosenqvist, 1990). Because all species have male pregnancy and many species are sex-role reversed, several studies have examined whether syngnathids deviate from the typical teleost steroid hormone profile with respect to the major sex steroids, testosterone (T)/11-ketotestosterone (KT) and estradiol ($E_2$) (Mayer *et al.*, 1993; Poortenaar *et al.*, 2004). Although endocrine studies on syngnathids are scant, there is currently little support for this hypothesis. Mayer et al. (1993) measured several androgens and $E_2$ in sexually mature adults of three species of pipefish (*S. typhle*, *S. acus*, and *Nerophis ophidion*). Despite the reversed sex roles observed in these species (Vincent *et al.*, 1995), the steroid hormone profiles of T/KT and $E_2$ were similar to those of fish species with conventional sex roles (Borg, 1994; Lokman et al., 2002b). However, one androgen, 11β-hydroxyandrostenedione (OHA) did not fit the typical teleost sex steroid profile. Within females, OHA was the sex steroid found in the highest concentration in the plasma; OHA levels were two-fold to eight-fold higher than $E_2$ and were comparable to those found in males (Mayer *et al.*, 1993). Female *S. scovelli*, a highly polyandrous, sex-role reversed species (Jones and Avise, 1997), also had higher levels of plasma OHA than that of T, KT or $E_2$ (Scobell *et al.*, *In prep*). When the plasma levels of competing pairs of females were examined, winning females had higher levels of OHA (but not T, KT, or $E_2$) than did losers. In comparison to other teleosts, syngnathids appear to be unique in that OHA is the highest plasma steroid in females (Lokman et al., 2002b).

Therefore, OHA is a likely candidate hormone for mediating female intrasexual competitive behavior in sex-role reversed syngnathids.

Little is known about the role of OHA in physiological or behavioral processes (Khan *et al.*, 1997). It is often thought of as a weak androgen (Lokman et al., 2002b; Suzuki et al., 2000), but OHA is used commonly in aquaculture to produce all-male stocks from genetic female fish fry (Baron et al., 2008a; Baron et al., 2008b; Baron et al., 2007; Desprez et al., 2003; Govoroun et al., 2001; Vizziano et al., 2008). The resultant males have functional testes, but the development occurs via a different pattern of gene expression than in genetic males (Baron et al., 2008a; Baron et al., 2008b; Baron et al., 2007; Govoroun et al., 2001). Under natural conditions, OHA may provide a pathway for masculinization of behavior without gonadal sex reversal. In mature female Japanese eels (*Anguilla japonica*) and rainbow trout (*Oncorhynchus mykiss*), OHA can be synthesized in the ovaries, and the amount of OHA that the follicles produce increases with developmental stage (Kazeto et al., 2011; Reddy et al., 1999). If OHA is produced by the ovaries in syngnathids, their asynchronous, continuous gamete production (Begovac and Wallace, 1987; Selman et al., 1991; Sogabe et al., 2008) may explain why plasma levels of OHA are high. The ovary in *S. scovelli* contains specific cohorts of gametes at progressive stages of maturity (Begovac and Wallace, 1988). When the most mature cohort of eggs move into the lumen during ovulation, the next cohort moves into its place and completes the final stage of maturation. If OHA is involved in the maturation of ova in syngnathids, it would likely be synthesized at relatively constant, high levels to meet the demands of continuous ova production. This

readily available source of plasma OHA could modulate both reproductive function and behavior through feedback mechanisms within the central nervous system. Immunoreactive OHA was found in pituitary gonadotropes of female African sharptooth catfish (Clariidae: *Clarias gariepinus*), demonstrating that this hypothetical feedback network is possible in female fish (Peute *et al.*, 1989).

Recent studies have shown that KT is also produced by the ovaries of mature female fish (Alam et al., 2005; Kazeto et al., 2011; Semenkova et al., 2006). Kazeto et al. (2011) report that female Japanese eels (*Anguilla japonica*) have high plasma levels of KT, and the most likely source is synthesis in the ovary from an androstenedione precursor with OHA as an intermediate product (Fig. 3.1). Conversion of OHA to KT gradually increased with consecutive stages of ovarian growth: OHA production from androstenedione increased to peak levels during mid-vitellogenic stages and remained high through late vitellogenesis. Ketotestosterone in the ovary of Japanese eels stimulates the oocytes to take up lipids and results in an increase in the oocyte diameter (Endo et al., 2010; Lokman et al., 2007). A similar increase in oocyte diameter in the presence of KT has also been observed in Atlantic cod (*Gadus morhua L.*) (Kortner et al., 2009; Kortner et al., 2008). In male fish, KT is a major androgen produced in the testis (Borg, 1994; Consten *et al.*, 2001) and regulates spermatogenesis (De Waal et al., 2008; Khan et al., 1997; Schulz and Miura, 2002). Ketotestosterone also induces male secondary sexual traits and mediates courtship and aggressive behavior (Borg, 1994; Goncalves and Oliveira, 2011; Munakata and Kobayashi, 2010). In females, the role of KT in the expression of these traits is less clear. However, implant studies have shown

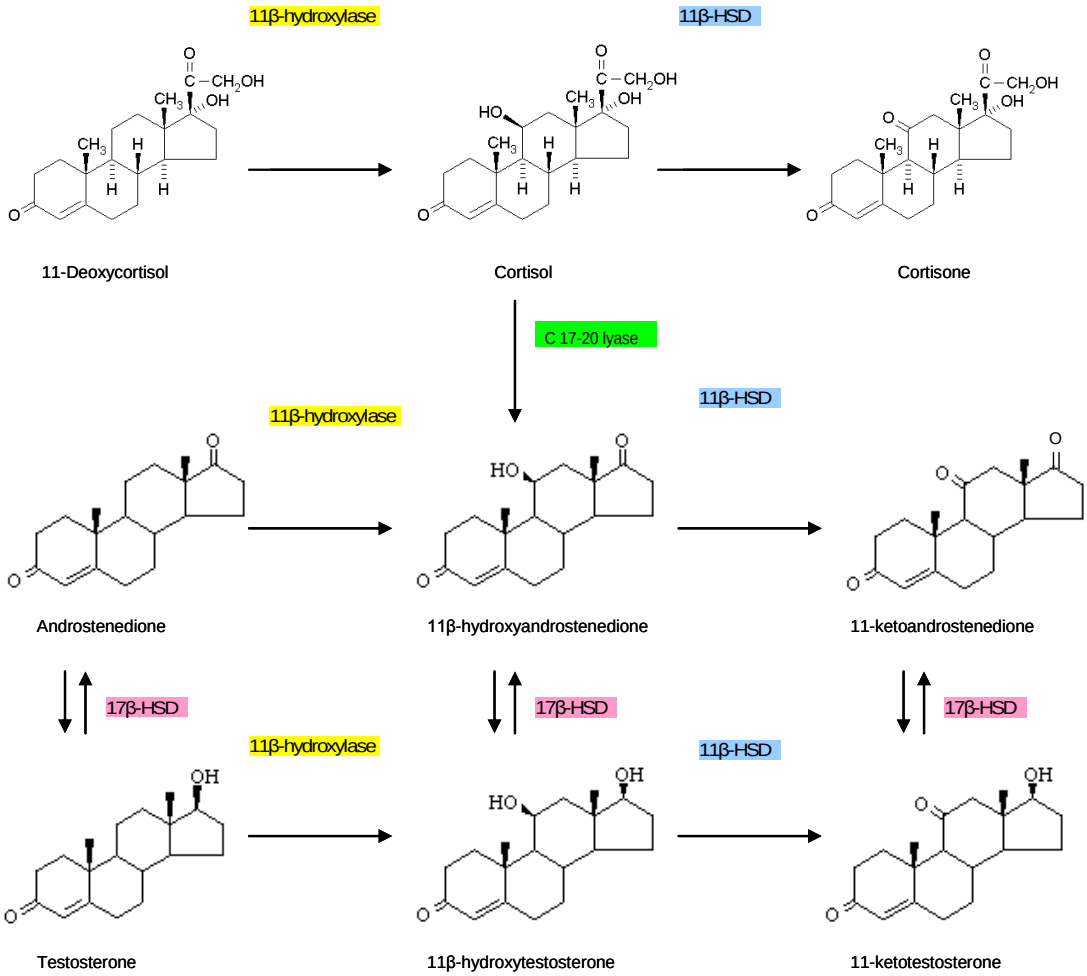



Fig. 3.1. Synthetic pathway of several androgens and glucocorticoids that use the enzymes 11β-hydroxylase and 11β-hydroxysteroid dehydrogenase.

that KT can masculinize female behavior and morphology. Female goldfish (*Carassius auratus*), gynogenetic crucian carp (*Carassius auratus langsdorfii*), and protogynous bluehead wrasse (*Thalassoma bifasciatum*) implanted with KT showed male-typical courtship towards stimulus females (Kobayashi and Nakanishi, 1999; Semsar and Godwin, 2004; Stacey and Kobayashi, 1996), yet KT did not inhibit female-typical reproductive behavior (Kobayashi and Nakanishi, 1999; Stacey and Kobayashi, 1996). These studies suggest that KT has the potential to promote male-typical reproductive behavior in females without behavioral defeminization. If sex-role reversed female syngnathids are accessing a male-typical neural network with androgens to increase aggression, KT is a likely mediator of this behavior.

Along with androgens, glucocorticoids are also often involved in the outcome of competitive contests (Briffa and Sneddon, 2007). Glucocorticoids could be an important factor in intrasexual female competition in *S. scovelli* because of the relationship between cortisol (the predominant glucocorticoid in most fish) and OHA. Human granulosa cells incubated with cortisol *in vitro* produced OHA, likely through side chain cleavage of cortisol (Fig. 3.1; Holownia et al., 1992). In addition, the release of OHA from the adrenals seems to be under similar control as cortisol. Injections of adrenocorticotropic hormone (ACTH) in guinea pigs, which predictably caused the release of cortisol into circulation, also caused a two-fold increase in plasma OHA concentrations and remained above baseline for six hours (Belanger *et al.*, 1993). In fact, the secretion profile of OHA over the six-hour period roughly mirrors that of cortisol. A similar positive correlation between plasma cortisol and OHA levels has been found in

female fish (Lokman et al., 2002b). It is also possible that all three hormones, (cortisol, OHA, and KT) are interrelated. Consten et al. (2002) showed that injections of cortisol in male common carp (*Cyprinus carpio*) caused a 400% elevation of plasma OHA within the first 30 minutes and a 30-40% increase in plasma KT by 2 hours post-injection. This study suggests that OHA may serve as a buffer for a muted KT response which results from elevated plasma cortisol during an intrasexual female aggressive interaction in *S. scovelli*.

Although one or more of these physiological mechanisms may allow for hormonal mediation of female aggression without inhibiting reproductive function, none of these alternative explanations has been tested. We used the sex-role reversed Gulf pipefish, *Syngnathus scovelli* (Evermann and Kendall 1896) as a model to examine the effect of exogenous hormone administration on intrasexual competitive behavior. We examined three alternative hypotheses versus the null hypothesis that OHA, KT, and cortisol have no effect on behavior. $H_1$: OHA is the principal mediator of female aggressive behavior; we predicted that female competitive behavior would increase with OHA treatment. $H_2$: KT is the principal mediator of female aggressive behavior, and OHA serves as a readily available precursor to KT. Here, we predicted that female competitive behavior would increase with KT treatment and would be greater than that observed in OHA-treated females. $H_3$: Cortisol mediates female competitive behavior, and OHA is a by-product of the activation of the stress response. We predicted that an acute elevation in cortisol would result in an increase of female competitive behavior.

## Methods

### *Collection and husbandry*

Sexually mature female *S. scovelli* (*N = 231*) were collected from Corpus Christi Bay, Texas, between July 30, 2007, and November 18, 2007. Pipefish were collected from sea grass beds by seine (1 x 2 $m^2$ with 2$mm^2$ nylon mesh). They were transported to Texas A&M University (College Station, TX, USA) in coolers with seawater from the field and bubblers that aerated the water. The water was treated with Stress Coat® to protect the fish slime coat and help to prevent infection. The fish were kept in coolers and allowed to acclimatize to laboratory conditions for 1-8 days (median 2 days) before the start of the experiment. Prior to placement in tanks, fish received a 10-minute freshwater dip to remove any external parasites. Females were housed under two different conditions depending on their role in the study. Test females were housed individually in 9.5 L tanks with a separate biological sponge filter in each that was supplemented periodically with saltwater bacteria. Opaque barriers were placed between tanks to prevent competitive interactions with other females. Thus, test females were physically, visually, and chemically isolated from other females until the day of the trial. In contrast, stimulus females were group housed (2-10 fish per tank) in 75.7 L tanks connected on a recirculating system. The system had a Bio Ball biological filter and each tank had an undergravel filter beneath the crushed coral substrate. Stimulus females were not kept in isolation because previous observations suggest that group housing results in

social subordination (SKS unpublished data). These two housing situations were designed to give the test female a competitive advantage and minimize the variance in stimulus female behavior. In both types of housing, plastic plants that mimic sea grass and 15 cm x 3 cm sections of grey PVC tubing cut in half were provided for refuge. All fish were fed twice daily with *Artemia* nauplii (www.brineshripdirect.com) enriched with Algamac Enhance® and Algamac ARA® (Aquafauna Bio-Marine, Inc., Hawthorne, CA, www.aquafauna.com). Fish were maintained under these conditions for 3-22 days (median 10 days) until being transferred to trial tanks. Ambient temperature was kept at 25 ºC and the L:D cycle at 14:10 h for the duration of the experiment.

*Behavioral trials*

Trials were run between August 24 and December 3, 2007, ($n$ = 20 replicates). The evening prior to the behavioral test, test females were lightly anesthetized with a 0.06% clove oil solution and given a 50 µl injection of a hormone solution (2 µg/ml of cortisol, OHA, or KT) or the vehicle (sesame seed oil) into the intraperitoneal cavity. Pressure was applied to the injection site following removal of the needle for roughly 30 seconds to ensure no injection solution was lost through the injection site. Two control groups of fish were handled similarly to those in the treatment groups, but did not receive anesthesia or an injection: competition, but no injection (non-injected control), and no competition, no injection (singletons). Fish were then placed in the test tank and allowed to recover from the anesthesia. After 20-30 minutes, injected fish were able to swim

normally. Trials were conducted the following day 11-24 hours post injection (median 17 hours) between 9:37 AM and 7:07 PM (median trial time 2:08 PM). Prior to the start of the trial, video recording began and a stimulus female was placed in the tank and given time to acclimatize to the test tank and the test female (acclimatization time range: 15-42 minutes; median: 24 minutes). In this resident-intruder paradigm, the stimulus female was usually immobile for the first few minutes of the acclimatization period. When the stimulus female began exploring the test tank, the trial started and scoring began. Test and stimulus female competitive behavior (Table 3.1) was scored in real time by SKS using BehaviorTracker software (www.behaviortracker.com) for 30 minutes.

Immediately following the trial, the test female was anesthetized with clove oil (0.04 mg/ml artificial saltwater) for 1 minute and a blood sample was taken from the caudal vein via a heparinized syringe fitted with a 26-gauge needle within 5 minutes of capture. Fish were then transferred to a terminal concentration of MS-222 (1 mg/ml artificial saltwater; Research Organics #1347A). The same procedure was used for stimulus fish after completing the blood draw from the test fish. The blood was centrifuged and plasma was drawn off, placed in a new 0.5 ml microcentrifuge tube, and frozen until EIA analysis. Following the last trial of the day, all test and stimulus females were measured for total length (TL), snout-vent length (SVL), body depth just anterior to the dorsal fin (standard depth, $Depth_{std}$) and at the deepest part of the trunk (maximum depth, $Depth_{max}$), and total mass ($Mass_T$), and gonads were dissected and weighed (wet

Table 3.1. Descriptions of female competitive behaviors recorded with BehaviorTracker software.

| Behavior | Type | Description |
|---|---|---|
| Approach | Frequency | The female moves more than 1 cm in the direction of the partner while facing her. |
| Lateral | Duration | The female is parallel to the partner with her side lateral to the other female's side; includes posing and dancing. |
| Lean | Frequency | The female leans her trunk toward the partner while being within 5 cm of her. |
| Ornament display | Duration | The female displays a change in partial or whole body coloration - black and white vertical striped banding. |
| Retreat | Frequency | While being within 10 cm of the partner, the female moves in a direction away from her partner. |
| S-shape | Duration | The female arches her trunk, pushing the belly outward and making the body look similar to an "s". |
| Touch | Frequency | The female touches a part of her body (usually the trunk) against the partner. |
| Twitch | Frequency | The female rapidly shakes her body laterally . |

mass, $Mass_G$). All tissues were preserved in 95% ethanol for potential DNA analysis later.

The procedures used in this study were approved by the Texas A&M University Research Compliance Institutional Animal Care and Use Committee (AUP #2007-242).

*Hormone assay*

A subset of test females were analyzed for plasma KT levels following the trial (n: KT-injected females = 16, non-injected singles = 6, non-injected controls = 5, oil-injected controls = 6). Ketotestosterone levels were measured with a commercial enzyme immunoassay (EIA) kit (Cayman Chemical #582751). The manufacturer's instructions were modified slightly to accommodate the small plasma volume of *S. scovelli* (median 13 µl, range 5-37 µl). From a subset of KT-injected, oil-injected (sham), no injection control, no injection and no competition control females, 10 µl of plasma was diluted with 490 µl of PBS and extracted four times with 2 ml ethyl acetate/hexane (50:50). The organic phase was decanted into a clean borosilicate test tube following freezing in a ¯80 ºC freezer for 5 - 10 minutes. Samples were dried down under nitrogen in a hot water bath and resuspended in 110 µl of EIA buffer (11:1 dilution). Samples and standard curve were run in duplicate, and the plate was incubated at 4 ºC overnight to increase the sensitivity of the assay. The next day, the plate was washed, developed, and read at 405 nm on a Vmax Kinetic ELISA Microplate Reader (Molecular Devices, www.moleculardevices.com) according to the manufacturer's instructions. The

sensitivity of the assay was 1.3 pg/ml (manufacturer's data) and the intra- and inter-assay coefficients of variation were 11% (manufacturer's data) and 10.4%, respectively. Cross reactivity was 2.9% for 11-ketoandrostendione, <0.01% for testosterone (manufacturer's data, respectively) and 1.8% for 11β-hydroxyandrostenedione. Due to the limited amount of plasma that can be obtained from *S. scovelli*, we did not measure plasma cortisol or OHA.

*Statistical analyses*

The resident-intruder paradigm and the group housing of the stimulus females resulted in several trials in which the stimulus female failed to interact with the test female. These trials were removed from the data set, and the remainder of the trials (n = 57) were used in analyses. The frequencies of the behaviors "lean," "twitch," and "touch" were combined. All behaviors were then square root transformed and entered into a principal components analysis. Principal component axis 1 (PCA1 behavior) explained 61.7% of the total variance in behavior. Body size scores were also obtained using a principal components analysis with the variables "total length," "total mass," and "standard depth." Principal component axis 1 (PCA1 body size) explained 88.3% of the total variance in body size. The five largest females in each treatment based on PCA1 body size were designated as "large" and the five smallest females were designated as "small." Females in the middle range of sizes that were not placed in one of these two categories were omitted (for each of KT, OHA, and oil-injected groups: n = 1, and for

cortisol: n = 4 females omitted) to maximize the difference between the body size of large and small groups and to obtain equal sample sizes in each treatment. Small and large test females' behavior across treatments was analyzed with a two-way ANOVA. Test females' PCA1 behavior scores were normally distributed and had homogeneity of variance across treatments and sizes. Post-hoc comparisons of groups were performed using a Tukey HSD analysis. To analyze the relationship between body size and behavior for the oil-, KT-, and OHA-injected groups further, a linear regression was performed for all females in each treatment group. For the subset of females for which plasma KT levels were measured, principal component axis 1 scores were obtained for body size and behavior as described above. Large and small females were designated for control and KT-injected females as described above, although no mid-sized females were omitted for this analysis. Two females, one KT-injected and one non-injected control female, had intra-assay coefficients of variation > 15% and were excluded from analyses. An outlier for the KT-injected group was removed and an ANCOVA was performed for large and small females' PCA1 behavior scores with measured plasma KT levels as a covariate and treatment group (control vs. KT-injected) nested within plasma KT levels. All analyses were performed using JMP 8.0.2 and $\alpha = 0.05$.

## Results

*Hormone effects on competitive behavior*

A two-way ANOVA (full model: $R^2 = 0.51$, $F_{9,49} = 4.69$, $p = 0.0003$; Fig. 3.2) revealed significant main effects of hormone treatment ($F_{4,49} = 4.01$, $p = 0.008$) and body size ($F_{1,49} = 11.14$, $p = 0.002$) on intrasexual female competitive behavior. There was also a significant interaction of treatment and body size on competitive behavior ($F_{4,49} = 3.77$, $p = 0.01$). The parameter estimates showed that competitive behavior was increased in large females in the KT treatment group (KT*large: $t = 2.74$, $p = 0.009$) and decreased in large females in the OHA treatment group (OHA*large: $t = -2.40$, $p = 0.02$). Post-hoc comparisons revealed that large KT-treated females performed more competitive behavior than small KT-treated females (Tukey HSD: $p = 0.01$). Large KT-treated females also performed more competitive behavior than small oil-injected (Tukey HSD: $p = 0.048$) and small non-injected control females (Tukey HSD: $p = 0.03$), as well as both small and large cortisol-injected females (Tukey HSD: small $p = 0.004$, large $p = 0.003$). Large oil-injected females also performed more competitive behavior than both small and large cortisol-injected females (Tukey HSD: small $p = 0.02$, large $p = 0.01$) and small KT-treated females (Tukey HSD: $p = 0.04$). All other comparisons were non-significant (Tukey HSD: $p > 0.05$).

A linear regression analysis showed that the body size of KT-injected females (Fig. 3.3) explained 58% of the variance in competitive behavior ($R^2 = 0.58$, $p = 0.006$). There

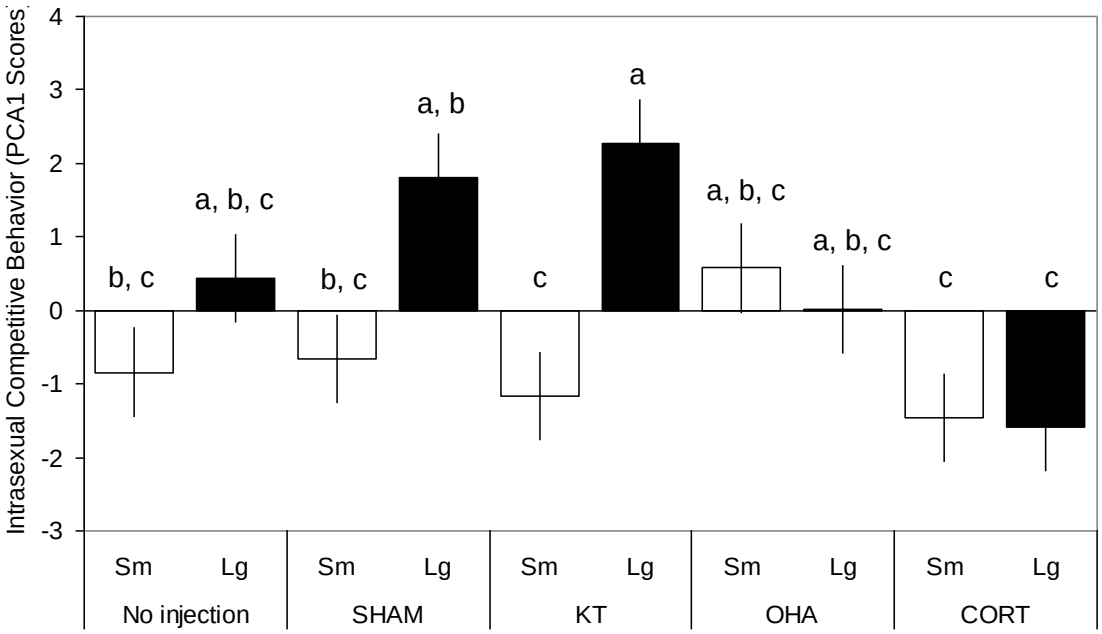



Fig. 3.2. Effect of hormone injection on female intrasexual competitive behavior (mean +/- SE of principal component). KT increased competitive behavior in large vs. small females. Cortisol decreased competitive behavior in all treated females.

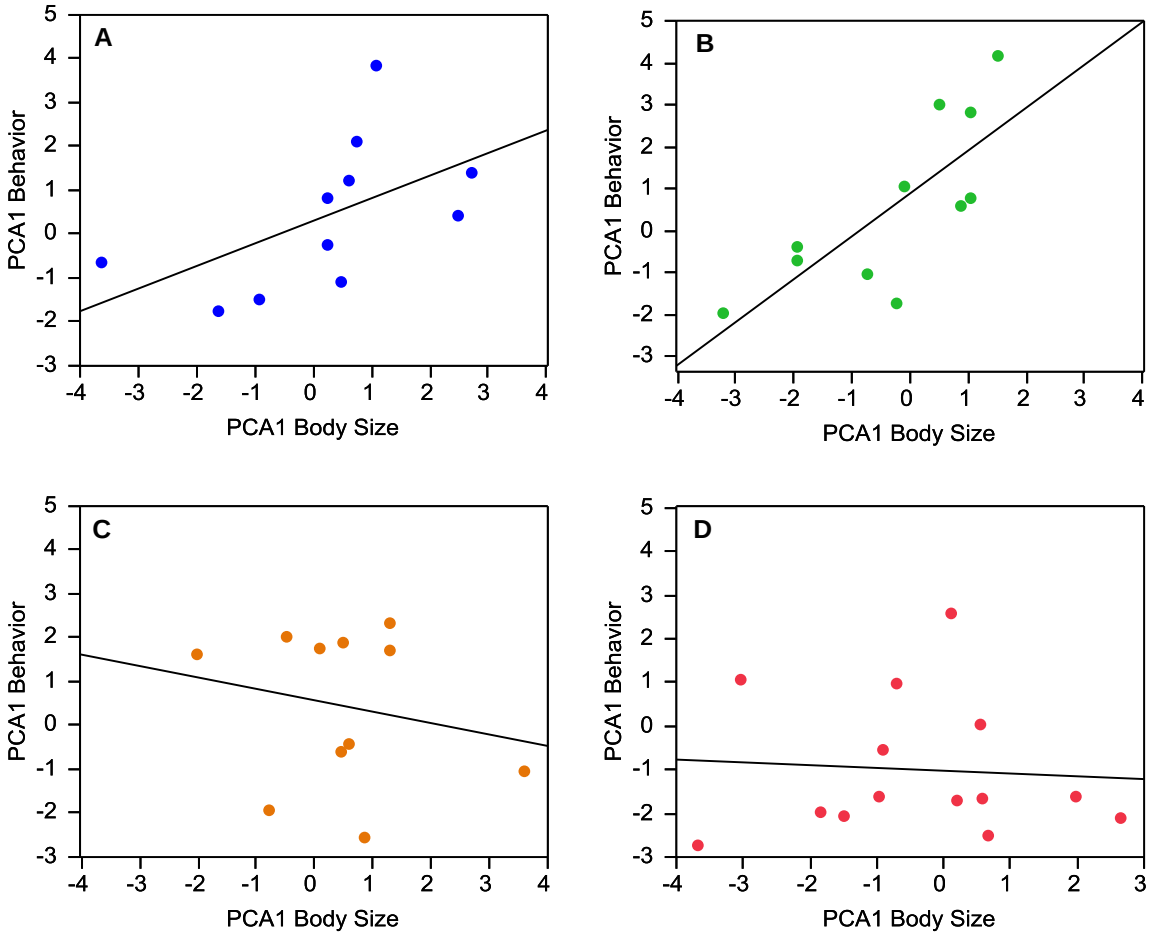



Fig. 3.3. Effect of body size on intrasexual competitive behavior for KT, oil, OHA and cortisol-injected females. (A) Competitive behavior tends to increase in oil-injected females ($R^2 = 0.30$, $p = 0.08$). (B) Larger KT-treated females perform significantly more competitive behavior ($R^2 = 0.58$, $p = 0.006$). (C,D) Competitive behavior in OHA-treated ($R^2 = 0.04$, $p = 0.54$) and cortisol-treated ($R^2 = 0.004$, $p = 0.82$) females shows no relationship to body size.

was a trend towards a positive effect of body size on competitive behavior in the oil-injected control females ($R^2 = 0.30$, $p = 0.08$). There was no significant relationship between these variables for the OHA-injected females ($R^2 = 0.04$, $p = 0.54$), cortisol-injected females ($R^2 = 0.004$, $p = 0.82$), or non-injected control females ($R^2 = 0.27$, $p = 0.13$; data not shown).

*Measured plasma KT levels and behavior*

There was no effect of competition on plasma KT levels; non-injected single females that did not compete had similar KT levels (38.9 ± 3.0 pg/ml) to non-injected (38.9 ± 4.2 pg/ml) and oil-injected controls (39.7 ± 3.1 pg/ml) that did compete (Kruskal-Wallis: $X^2 = .06$, $p = 0.97$). Because they did not differ, these three control groups were combined, and plasma KT levels were compared to those of the KT-injected females. Plasma KT levels were higher in the KT-injected group (44.9 ± 3.1 pg/ml) than in the control group (39.2 ± 1.8 pg/ml; median test: $X^2 = 5.9$, $p = 0.01$). However, this was due to one outlier in the KT-injected group; when this female was removed from the analysis, KT levels did not differ (KT-injected: 42.1 ± 1.3 pg/ml; controls: 39.2 ± 1.8 pg/ml; t-test: $t_{27} = 1.29$, $p = 0.21$). Without this outlier, there was also no effect of the time of the trial (full model: $R^2 = 0.10$, $F_{3,26} = 0.83$, $p = 0.49$) or the time between the injection and the trial (full model: $R^2 = 0.11$, $F_{3,26} = 0.95$, $p = 0.43$) on plasma KT levels in control and KT-injected females.

The effect of plasma KT levels on competitive behavior (PCA1 behavior) was compared between large and small females for either KT-injected or control females (oil-injected and non-injected controls). An ANCOVA (full model: $R^2 = 0.77$, $F_{5,16} = 7.19$, $p = 0.003$; Fig. 3.4) revealed a significant main effect of body size ($F_{1,16} = 15.12$, $p = 0.003$) and an interaction between body size and plasma KT levels ($F_{2,16} = 4.97$, $p = 0.03$) on female intrasexual competitive behavior. Large females showed an increase in competitive behavior with higher plasma KT levels, whereas small females did not. The parameter estimates showed that competitive behavior within treatment groups increased with plasma KT levels in large KT-injected females (Group[KT]: plasma KT*body size[L]: $t = 2.67$, $p = 0.02$), but not large control females (Group[control]: plasma KT*body size[L]: $t = 1.50$, $p = 0.16$) when compared to small females within their treatment group.

**Discussion**

Sex role reversed competitive behavior in syngnathids may have evolved through the exploitation of ancestral hormonal mechanisms that had originally evolved in males (Ketterson, 2007). If such a process occurred, it would have only done so in a manner that did not inhibit reproductive function to such a degree as to be detrimental to the overall fitness of the organism. Assuming sex role reversed competitive behavior in female syngnathids occurs via a masculinization process, it is likely mediated via less conventional mechanisms than those that occur in other male teleosts. We examined

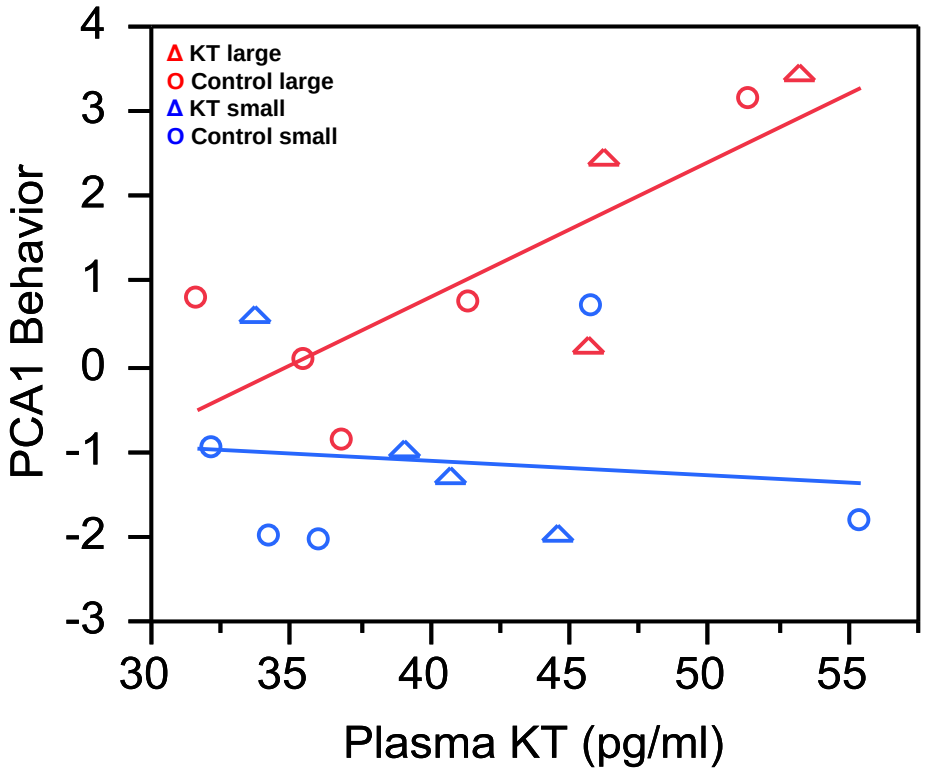



Fig. 3.4. Effect of circulating plasma KT levels on intrasexual competitive behavior in large and small KT-treated and control females. Plasma KT levels have opposite effects on competitive behavior in large vs. small KT-injected females (Group[KT]: plasma KT*body size[L]: $t = 2.67$, $p = 0.02$), but not large vs. small control females (Group[control]: plasma KT*body size[L]: $t = 1.50$, $p = 0.16$).

three steroids that are interconnected in the steroid synthesis pathway and have all been implicated in masculinization in fish. We tested the alternative hypotheses that either OHA, KT, or cortisol was the principle mediator of intrasexual competitive behavior in *S. scovelli*.

Despite OHA being the highest sex steroid measured in the plasma of female *S. scovelli* (Scobell et al., In prep), KT treatment showed the greatest effect on female competitive behavior. KT interacted with body size to enhance the difference in competitive aggression between large and small females. Ketotestosterone treatment resulted in a significant positive effect of body size on competitive behavior. Higher levels of circulating plasma KT increased competitive behavior in large females treated with KT, but did not do so in small KT-treated females. This trend also appeared in the control females (sham- and no-injection), but was not significant. Thus, treatment with KT strengthened the effect of body size on competitive behavior in *S. scovelli.* In a previous study where females were unaltered prior to a dyadic interaction and were tested shortly after collection from the field (24-48 hours), competitive behavior did show a significant positive increase with larger body size (Scobell *et al.*, *In prep*). The data from the current experiment suggests that this relationship is likely KT-dependent. Large body size in females affects both pre- and post-copulatory sexual selection in *S. scovelli* (Jones et al., 2001a; Paczolt and Jones, 2010). Males prefer to mate with large females and successfully brood a greater number of embryos from them (Paczolt and Jones, 2010). Also, several other species of syngnathids have shown that the presence of large, dominant females can result in reproductive costs to small, subordinate females

(Berglund and Rosenqvist, 2003; Bernet et al., 1998; Rosenqvist, 1990) to the extent that females may reduce reproductive effort to invest energy in growth (Berglund, 1991). This could be a strategy associated with life history because smaller females are likely also younger females in fish with indeterminate growth. It follows that sexual selection would act to integrate body size with competitive behavior via a hormonal mechanism such that females make optimal life history choices for a given size. What is still to be elucidated is how KT differentially mediates competitive behavior in large and small females.

Also of interest is whether KT mediates intrasexual competitive behavior acting as a physiological trigger, a primer as testosterone does in males (Briffa and Sneddon, 2007), or as a potentiator in large, but not small females (Munakata and Kobayashi, 2010). Circulating KT levels in large females have a dose-dependent effect on the relationship between body size and competitive behavior, which suggests KT is acting like a physiological trigger. However, because the injections were given the evening prior to the trial and the measured KT levels were not different from those of controls following the trial, a priming or potentiating effect of KT cannot be ruled out (Briffa and Sneddon, 2007). It is conceivable that the injections of KT were ineffective in producing elevated plasma KT levels, but we think this possibility is unlikely. Care was taken while giving the injection to make sure the injection site was closed before releasing the fish back into the water and no injection solution was observed escaping from the injection site. We think it is more likely that the KT was metabolized by the time the blood sample was taken. In a separate experiment (see Chapter IV), we have shown that injections of KT

(dissolved in a saline vehicle) elevate plasma KT levels for 1 hour, and then plasma KT returns to near-baseline levels. In support of this idea is the fact that the outlier female with the highest measured plasma KT levels had the shortest time in between the injection and the blood collection (11.5 hrs.) of the KT-injected females.

Our alternative hypothesis that cortisol mediates female competitive behavior was supported, although our prediction regarding the direction of the response was incorrect. Competitive behavior decreased in both large and small female *S. scovelli* treated with cortisol. This reduction in competitive behavior with cortisol treatment is reminiscent of the relationship between behavior and glucocorticoid levels in socially subordinated animals. Subordinate animals frequently have reduced aggressive behavior that correlates with elevated levels of glucocorticoids that persist days or weeks following social subordination (Briffa and Sneddon, 2007; Oliveira and Goncalves, 2008). On the other hand, dominant animals often show an increase in aggressive behavior that correlates with an acute elevation in glucocorticoids that return to baseline levels within hours of an encounter. Our cortisol treatment seems to have mimicked a social subordination situation with respect to intrasexual competitive behavior. It is possible that a cortisol treatment regime that mimicked the acute increase in plasma levels seen in winning or dominant animals would increase aggressive behavior in *S. scovelli* females. In addition to decreasing overall competitive behavior, cortisol treatment disrupted the positive relationship between body size and competitive behavior in KT-treated females (and the trend for such a relationship in oil-injected females). Large females injected with cortisol were no more likely than small female to show high levels of competitive

behavior. Thus, large females undergoing chronic stress and sustaining elevated cortisol levels for an extended period would lose their competitive edge. It is possible that group housing was such a stress for the stimulus females in this experiment and that explains their social subordination and resultant diminished competitive behavior.

Our hypothesis was not supported with respect to OHA. Despite the fact that OHA is the highest sex steroid measured in female *S. scovelli* and is higher in the plasma of winning than losing females following a short-term competition (Scobell *et al.*, *In prep*), there was no increase in competitive behavior in OHA-treated females. The parameter estimates from the two-way ANOVA suggested that there was a negative relationship between body size and competitive behavior in OHA-treated females. However, the linear regression showed no relationship between these variables when OHA-treated females were considered alone. OHA treatment resulted in intermediate levels of competitive behavior that were not different from any other treatment group. These results suggest that OHA is not the hormone directly mediating female intrasexual behavior. In addition, because OHA did not show a similar pattern between body size and competitive behavior as KT, our results do not support a mechanism by which OHA is converted into KT to mediate competitive behavior. In fact, the effect of body size on competitive behavior seems to be disrupted by OHA in a similar manner to that of cortisol.

It is possible that the high plasma levels of OHA observed previously in *S. scovelli* (Scobell et al., *In prep*) and other pipefish (Mayer *et al.*, 1993) reflect this hormone's relationship to cortisol. Assuming that the release of OHA from the adrenals in *S.*

*scovelli* is under similar control to cortisol as it is in the guinea pig (Belanger *et al.*, 1993), activation of the hypothalamic-pituitary-adrenal (HPA) axis during an intrasexual competition could result in an increase in both plasma cortisol and OHA concentrations. Thus, high plasma OHA levels could reflect the results of a fight-or-flight response. Alternatively, OHA could be a product of ovarian maturation with little to no effect on competitive behavior. In male mammals, OHA seems to have low androgen potency (Belanger *et al.*, 1993; Suzuki *et al.*, 2000). Although OHA is produced in the testis of both Japanese eels (*Anguilla japonica*) and zebrafish (*Danio rerio*), it has low competitive binding for the androgen receptor(s) and in zebrafish, OHA did not induce functional transactivation of the androgen receptor (De Waal et al., 2008; Ikeuchi et al., 1999). Similar to the interaction with the androgen receptor in male fish, OHA showed almost no competitive binding with the putative androgen receptor in coho salmon (*Oncorhynchus kisutch*)(Fitzpatrick *et al.*, 1994).

If OHA is a relatively non-reactive metabolite in the androgen synthesis pathway in syngnathids, it could serve a pivotal role in providing a source of androgen precursor for KT synthesis while protecting females from excessive masculinization via elevated levels of more active androgens. Exogenous T induced a rudimentary male-like brood pouch in female *S. schlegeli* (Noumura, 1959) and female long-snouted seahorses (*Hippocampus hippocampus*)(Boisseau, 1967a). The ovary that was in direct contact with a testosterone pellet was smaller than the other ovary, had a reduced ovarian cavity, a thickened ovarian wall, and few mature eggs in female *S. schlegeli* (Noumura, 1959). These studies show that high levels of T can lead to a reduction in fecundity and external

masculinization that would result in decreased fitness. The synthesis of KT via OHA instead of T (Fig. 3.1) could represent a pathway via which females can achieve a balance of T, the substrate necessary for aromatization to $E_2$, and KT, the hormone that likely regulates oocyte maturation and female reproductive behavior. OHA could also serve as a buffer for cortisol to prevent its masculinizing effects. Several studies on environmental sex change have shown that fish are masculinized because cortisol shuts down aromatase (the enzyme that converts T to $E_2$) and other early ovarian differentiation genes (Hattori *et al.*, 2009; Hayashi *et al.*, 2010; Yamaguchi *et al.*, 2010). If cortisol can be deactivated by producing OHA via side chain cleavage, females could engage in intrasexual competitions without a concomitant decrease in fitness due to masculinization.

To what degree cortisol, OHA, and KT interact to mediate female intrasexual competition requires further study. Ideally, these three steroids should be measured for a female both before an intrasexual competition and after to know the change that is induced in each hormone by the interaction. This approach could be facilitated by measuring these steroids in fish-holding water. This technique allows for repeated sampling and has been validated in other fish for cortisol and KT (Kidd et al., 2010; Scott et al., 2008). Another approach would be to measure the changes in expression levels of the enzymes involved in the androgen synthesis pathway during different social interactions. A shift in enzyme expression could inform when each steroid is synthesized. In addition, comparing steroid hormone or enzyme expression levels in

syngnathids with different levels of intrasexual competition should help elucidate how such a unique female reproductive strategy evolved within this lineage.

# CHAPTER IV

## THE EFFECT OF 11-KETOTESTOSTERONE INJECTIONS ON FEMALE INTRASEXUAL COMPETITIVE BEHAVIOR IN *Syngnathus scovelli*

Androgens have been shown to mediate male reproductive behavior across vertebrate taxa. Androgens mediate courtship and copulatory behavior as well as male intrasexual aggression, territorial aggression, and rank-related aggression (Nelson, 2005). There have been relatively few studies on the role of androgens in mediating behavior in female vertebrates, but recent studies suggest they are involved in aggression and sexual behavior (Staub and De Beer, 1997). Female spotted sandpipers (*Actitus macularia*) that had obtained a mate had seven times higher testosterone levels than unpaired females (Fivizzani and Oring, 1986). Among female moorhens (*Gallinula chloropus*), heavier, dominant individuals had higher testosterone levels than lighter, subordinate females (Eens and Pinxten, 2000). Competition among female dunnocks (*Prunella modularis*) caused increases in fecal testosterone levels that were also correlated with increases in an aggressive call (Langmore *et al.*, 2002). In the same study, females placed in a polygynandrous setting (where multiple females competed for multiple males) also had higher testosterone levels than females in a monogamous setting. Female mountain spiny lizards (*Sceloporus jarrovi*) had high levels of testosterone during the part of the year when they were more aggressive compared with other times of year (Woodley and Moore, 1999). Female spotted hyenas (*Crocuta crocuta*) are socially aggressive and dominate males. Dominant females have higher

circulating androgen levels during late gestation than subordinate females (Dloniak *et al.*, 2006); cubs born to dominant females with high androgen levels show higher levels of aggression and mounting behavior themselves. A study of androgen levels in female ring-tailed lemurs (*Lemur catta*), a female-dominant species, showed a two-fold increase in both intrasexual conflict rates and androgen levels during the breeding season; however, females in the latest stage of follicular development had the lowest level of fecal androgens (Von Engelhardt et al., 2000). These studies suggest that androgens play a role in mediating female sexual behavior in a manner similar to that in males. Yet, in all of these species (with the exception of moorhens during the periods of the year when they are most aggressive) females have lower levels of circulating androgens than males.

Females may be constrained from having androgen levels as high as males due to differences in reproductive physiology. Experimentally elevated testosterone levels in dark-eyed juncos (*Junco hyemalis*) do increase intrasexual aggression, but also result in a longer interval between nest completion and appearance of the first egg, lower body mass, inhibition of brood patch development, decreased cell-mediated immune function, and a reduction in mate discrimination (Clotfelter et al., 2004; Mcglothlin et al., 2004; Zysling et al., 2006). Injections of testosterone in female zebra finches (*Taeniopygia guttata*) reduced clutch size and suppressed laying of the third and fourth eggs in the clutch (Rutkowska *et al.*, 2005). Whereas high androgen levels in males support reproductive structures and promote spermatogenesis, high doses of androgens in females can have negative side effects on reproduction, immunity, and mate choice.

Although female vertebrates in higher taxa may experience trade-offs between elevated androgen levels and fecundity, fish have a wide array of reproductive diversity (e.g. in sex determination mechanisms, in mating systems, and in reproductive tactics) and may be less constrained in this area (Desjardins and Fernald, 2009; Godwin, 2010). Mank (2007) showed that peak testosterone levels in female ray-finned fishes (Actinopterygii) were strongly positively correlated with male peak androgen levels in many taxa. Female fish frequently have testosterone levels that are equal to or greater than that of males of their species (Borg, 1994; Lokman et al., 2002a; Mank, 2007). The role of elevated androgens in teleost fish is poorly understood. Similar to studies in higher vertebrates, a few studies have shown that experimentally elevated androgens in female fish result in increases in aggressive behavior (Dulka and Maler, 1994; Munro and Pitcher, 1985). In the cooperatively breeding cichlid (*Neolamprologus pulcher*), females involved in aggressive encounters had higher levels of testosterone and 11-ketotestosterone (KT), a fish-specific androgen, than controls (Desjardins *et al.*, 2006). When compared to males, breeder females that defend territories against intruders had higher levels of testosterone, but lower levels of KT; helper females had similar levels of testosterone to males and lower levels of KT (Desjardins *et al.*, 2008). After acquiring a new territory, dominant females had higher levels of testosterone than did subordinate females; KT levels did not differ between the two groups (Taves *et al.*, 2009). In several species of teleost fish, exogenous KT has been shown to induce male-typical courtship. Female goldfish (*Carassius auratus*) implanted with KT produced the full suite of male-typical sexual behavior including courtship and spawning complete with the

stereotypical male ejaculatory act at levels comparable to males (Stacey and Kobayashi, 1996). A similar experiment in gibuna, a gynogenetic crucian carp (*Carassius langsdorfii*), produced complementary results in that KT-implanted females produced male-typical spawning behavior (Kobayashi and Nakanishi, 1999). In both species, androgen implants did not prevent females from displaying female-typical courtship behavior when it was induced via injections of prostaglandin $F_{2\alpha}$ (Kobayashi and Nakanishi, 1999; Stacey and Kobayashi, 1996). In the protandrous sex-changing bluehead wrasse (*Thalassoma bifasciatum*), plasma KT levels increase as the dominant female changes sex and assumes the role of a territorial male. Ovariectomized females implanted with KT capsules displayed male-typical skin coloration and courtship behavior (Semsar and Godwin, 2004). These studies suggest that sexual behavior in fish is plastic and that androgens can mediate sexual and aggressive behavior in female fish. However, further studies are needed to determine how androgens might mediate these behaviors across the variety of mating and social systems in teleosts.

Female Gulf pipefish (*Syngnathus scovelli*) perform intense ritualized aggressive displays when in the presence of other conspecific females and courtship displays when males are present. Unlike many other teleosts where these behavioral repertoires are different (Goncalves and Oliveira, 2011), intersexual courtship and intrasexual competitive displays in *S. scovelli* have similar behavioral sequences (see Chapter V). Considering that implants of KT in female carp induced male-like courtship without inhibiting female-typical sexual behavior (Kobayashi and Nakanishi, 1999; Stacey and Kobayashi, 1996), and female *S. scovelli* courtship and competitive behavior are very

similar (see Chapter V), it seems likely that female syngnathids may use KT to mediate intrasexual aggression. Our previous results suggested that KT treatment the evening prior to an intrasexual contest resulted in an increase in competitive behavior in large versus small females. In the current study, we wanted to know whether an acute elevation of KT would result in an increase in intrasexual competitive behavior. We first wanted to determine the time course of peak plasma KT elevation following an injection of a KT solution. Next, we wanted to determine whether female intrasexual competitive behavior would respond to KT treatment in a dose-dependent manner. We tested the hypothesis that KT activates female aggressive behavior in Gulf pipefish. We predicted KT would increase competitive aggression and that higher doses of KT would have a greater effect on female behavior.

## Methods

### *Experiment 1 – Plasma KT time course following injection*

Sexually mature female *S. scovelli* (n = 46) were collected from Corpus Christi Bay, Texas on May 22, 2008. Pipefish were collected by seine (1 x 2 $m^2$ with 2$mm^2$ nylon mesh) from sea grass beds. They were placed in coolers with seawater from the field and bubblers that aerated the water and were transported to Texas A&M University (College Station, TX, USA). The following day, fish were given a 10-minute freshwater dip to remove any external parasites and were then placed in aquaria. Fish were individually

housed in 9.5 L tanks with a separate biological sponge filter in each that was supplemented periodically with saltwater bacteria. Plastic plants that mimic sea grass were provided for refuge. Opaque barriers were placed between tanks to prevent competitive interactions with other females. Thus, females were physically, visually, and chemically isolated from other females. All fish were fed twice daily with *Artemia* nauplii (www.brineshripdirect.com) enriched with Algamac Enhance® and Algamac ARA® (Aquafauna Bio-Marine, Inc., Hawthorne, CA, www.aquafauna.com). Ambient temperature was kept at 25 ºC and the L:D cycle at 14:10 hours.

On May 30, 2008, females were lightly anesthetized with a 0.06% clove oil solution and given a 50 µl injection of a hormone solution (2 µg/ml of OHA or KT in phosphate buffered saline) into the intraperitoneal cavity. Pressure was applied to the injection site following removal of the needle for roughly 30 seconds to ensure no injection solution was lost through the injection site. Fish were randomly selected to be sampled at 1, 3, 5, 7, and 9 hours post-injection. Twelve fish did not survive the procedure and were not sampled for blood. Three females did not receive an injection; blood samples from these fish were used to obtain a baseline level of plasma KT (0 hours post-injection). At the designated time post-injection, each fish was lightly anesthetized with a 0.06% clove oil solution for 1 minute and a blood sample was taken from the caudal vein via a heparinized syringe fitted with a $26^{3/8}$-gauge needle within 5 minutes of capture. Fish were then transferred to an overdose solution of MS-222 (1 mg/ml artificial saltwater; Research Organics #1347A) until they succumbed to the agent. The blood sample was transferred to a 0.5 ml microcentrifuge tube. The blood was centrifuged and plasma was

drawn off the top, placed in a new 0.5 ml microcentrifuge tube, and frozen at -20 ºC until enzyme immunoassay (EIA) analysis. Fish were measured for total length (TL), snout-vent length (SVL), body depth just anterior to the dorsal fin (standard depth, $Depth_{std}$) and at the deepest part of the trunk (variable depth, $Depth_{max}$), and total mass ($Mass_T$), and gonads were dissected and weighed (wet mass, $Mass_G$).

*Experiment 2 – KT effects on competitive behavior*

Sexually mature female *S. scovelli* (n = 97) were collected from Corpus Christi Bay, Texas between July 12 and September 13, 2008, and were transported, housed, and fed as in Experiment 1. Trials were run between July 30 and September 18, 2008 (n = 14 replicates). Three experimental tanks (61 L, 50.8 x 25.4 x 45.7 cm) were divided in half by a permanent, sealed, opaque piece of Plexiglas® (Fig. 4.1). Each of these compartments was then divided by a removable opaque piece of Plexiglas®, which was used to separate the competing females as they acclimatized to the test tank. The evening prior to the behavioral test, a stimulus female and a test female were placed on either side of the opaque barrier in the designated compartment. The next morning on the day of the trial, test females were lightly anesthetized with a 0.06% clove oil solution and given a 50 µl injection of one of the following solutions into the intraperitoneal cavity: 0, 0.0125, 0.025, 0.05, 0.1, or 0.2 µg/ml of KT in phosphate buffered saline. Pressure was

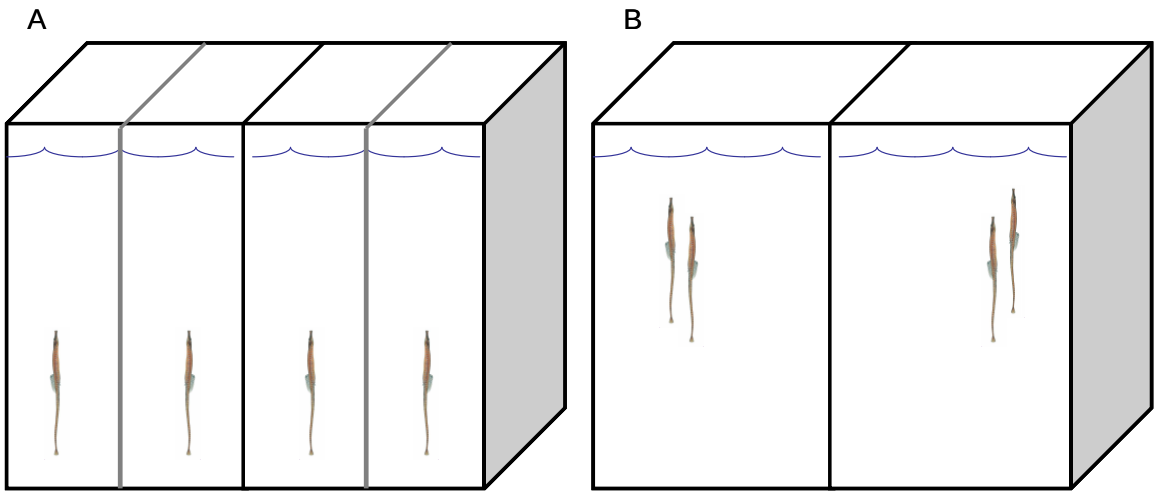



Fig.4.1. Experimental tank set up. (A) The 61 L tank is divided in half by a permanent opaque barrier (black); test and stimulus females are separated by a removable opaque barrier (grey). (B) At the start of the trial, the barrier separating pairs of females are removed and females are allowed to interact for 30 minutes.

applied to the injection site following removal of the needle for roughly 30 seconds to ensure no injection solution was lost through the injection site. Fish were then placed in the test tank and allowed to recover from the anesthesia for one hour; results from Experiment 1 indicated that one hour post-injection would produce peak plasma KT concentrations (see results below). After 20-30 minutes, injected fish were able to swim normally. Size matched stimulus females (average TL difference from test female = 2 mm) were not handled, anesthetized, or injected prior to the trial. Trials were started between 10:22 AM and 3:46 PM. At the start of the trial, video recording began, bubblers were removed from the experimental tanks, and the barriers were removed so that stimulus-test pairs could interact for 30 minutes. Immediately following the trial, the test female was removed from the test tank and a blood sample was collected (within 5 minutes of capture) and processed for EIA analysis as in Experiment 1. The test female was killed with an overdose of MS-222. Following the last trial of the day, all test females were measured (TL, SVL, $Depth_{std}$, $Depth_{max}$, $Mass_T$), and ovaries were dissected and weighed (wet mass). Stimulus females were returned to their individual 9.5 L tank and housed until they were used as test females on a later trial day (with the exception of the stimulus females from the last two trial days; these fish were killed and measured shortly following the trial).

Test and stimulus behavior (Table 4.1) was scored from video using BehaviorTracker software (www.behaviortracker.com) for 30 minutes. Scoring began when the barrier was removed from the second compartment (barriers in compartments

Table 4.1. Descriptions of behaviors recorded with BehaviorTracker software.

| Behavior | Type | Description |
|---|---|---|
| Ornament display | Duration | The female displays a change in partial or whole body coloration - black and white vertical striped banding. |
| Dancing | Duration | Female swims vertically with the body rigid with the abdomen pushed forward |
| Lean | Frequency | Female leans its head and trunk toward the other female while within 5 cm |
| Posing | Duration | Female has chest, but not tail, lifted off of the substrate at a 45 degree angle and the abdomen is pushed forward |
| Resting | Duration | Female is laying on the substrate or is motionless up against a wall not displaying |
| Swimming | Duration | Female is swimming around the tank not displaying or interacting with the partner |
| Touch | Frequency | Female makes physical contact with the partner female |
| Twitch | Frequency | Female rapidly shakes its body laterally |

within a tank had been removed one immediately following the next at the start of the trial). The trials for both compartments in a tank usually were scored simultaneously. However, in instances where there was a substantial amount of activity in both trials simultaneously, trials were scored separately to ensure accuracy. Behavior of the test animal was altered due to the anesthesia or the injection in several trials (n = 9); these trials were not scored.

The procedures used in these studies were approved by the Texas A&M University Division of Research and Graduate Studies Office of Research Compliance Institutional Animal Care and Use Committee (AUP #2007-242).

*Hormone assays*

Ketotestosterone levels were measured with a commercial EIA kit (Cayman Chemical #582751). The manufacturer's instructions were modified slightly to accommodate the small plasma volume of *S. scovelli* (median 15 µl, range 9-20 µl). Briefly, 10 µl of plasma from an individual fish (Experiment 1: n = 34 females, Experiment 2: n = 60 females - 10 females per treatment group) was diluted with 490 µl of phosphate buffered saline (PBS) and extracted four times with 2 ml ethyl acetate/hexane (50:50). The organic phase was decanted into a clean borosilicate test tube following freezing in a ¯80 ºC freezer for 5 - 10 minutes. Samples were dried down under nitrogen in a hot water bath and resuspended in 110 µl of EIA buffer (11:1 dilution). Samples and standard curve were run in duplicate and the plate was incubated

at 4 ºC overnight to increase the sensitivity of the assay. The next day, the plate was washed, developed, and read at 405 nm on a Vmax Kinetic ELISA Microplate Reader (Molecular Devices, www.moleculardevices.com) according to the manufacturer's instructions. The sensitivity of the assay was 1.3 pg/ml (manufacturer's data) and the intra- and inter-assay coefficients of variation were 11% (manufacturer's data) and 10.4%, respectively. Cross reactivity was 2.9% for 11-ketoandrostendione, <0.01% for testosterone (manufacturer's data, respectively) and 1.8% for 11β-hydroxyandrostenedione. For both experiments, many samples were above the range of the standard curve of the assay (1000 pg/ml) and were extrapolated using the Cayman EIA Analysis spreadsheet (http://www.caymanchem.com/app/template/analysis%2CEIA.vm).

*Statistical analyses*

In Experiment 1, plasma KT levels across sampling times for both KT- and OHA-injected female groups were compared with a Wilcoxon rank sums test. Post-hoc comparisons of groups were performed using a Tukey HSD analysis.

In Experiment 2, plasma KT concentrations following injections of differing doses of KT solutions were compared across treatment groups using a Wilcoxon rank sums test. Post-hoc comparisons of groups were performed using a Tukey HSD analysis. However, many of the plasma KT values for the higher treatment doses were above the range of the standard curve (1000 pg/ml) and were highly extrapolated values, which

resulted in large variances in these groups. Removing these off-the-curve values resulted in the deletion of all females in the 0.1 µg/ml KT and 0.2 µg/ml KT treatment groups as wells as four females in the 0.05 µg/ml KT group and three females in the 0.025 µg/ml KT groups. Plasma KT concentrations were compared across treatment groups for those samples that were on within the range of the assay using a Wilcoxon rank sums test and a Tukey HSD post-hoc analysis.

To determine the effects of KT injections on competitive behavior, durations and frequencies of behaviors were adjusted for the total trial time, which was slightly shorter than 30 minutes in several trials due to an inadvertent stop of the video recording (average trial time = 29:53 min.). A composite score for competitive behavior was obtained via a principal components analysis (PCA). To reduce the number of variables entered into the PCA, like variables were combined: the durations of "pose" and "dance," and "rest" and "swim" were combined as were the frequencies of "lean," "twitch," and "touch." Principal component axis 1 (PCA1) of these combined variables plus the duration of "ornament display" explained 83.5% and 79.1% of the total variance in behavior for test and stimulus females, respectively. Competitive behaviors (dance + pose, lean + twitch + touch, ornament display) loaded positively on PCA1, whereas non-interactive behavior (rest + swim) loaded negatively on PCA1 for both test and stimulus females. Therefore, higher positive PCA1 behavior scores can be interpreted as higher levels of competitive behavior and higher negative PCA1 behavior scores as lower levels of competitive behavior. Because the body size of interacting females affects the amount of competitive behavior displayed (Scobell *et al.*, *In prep*), the residuals of stimulus and

test females' PCA1 behavior scores regressed on the test female TL were used to determine the effect of treatment on competitive behavior in *S. scovelli* females. An ANCOVA was used to determine the effect of KT treatment on test female size-adjusted PCA1 behavior scores using stimulus female size-adjusted PCA1 behavior scores as a covariate. Residuals from this model were normally distributed and had similar variances among groups. A subset of females that had plasma KT levels within the physiological range (under 500 pg/ml; Scobell *et al.*, *In prep*) and a coefficient of variation under 15% (n = 18) were used in a multiple linear regression analysis that examined the effects of plasma KT, test female TL, and stimulus female PCA1 behavior scores on test female PCA1 behavior scores. All analyses were performed using JMP 8.0.2 and $\alpha = 0.05$.

**Results**

*Experiment 1*

Intraperitoneal injections of 2 µg/ml solutions of either OHA or KT resulted in a significant increase in plasma KT levels in female *S. scovelli* (Wilcoxon rank sums, OHA: $X^2 = 13.6$, $p = 0.02$; KT: $X^2 = 15.0$, $p = 0.01$; Fig. 4.2). Post-hoc analyses revealed that circulating KT was increased one hour post-injection in both the OHA and KT treatment groups when compared to all other time points (Tukey HSD, OHA: $p < 0.03$ and KT: $p < 0.0001$ for all comparisons, respectively).

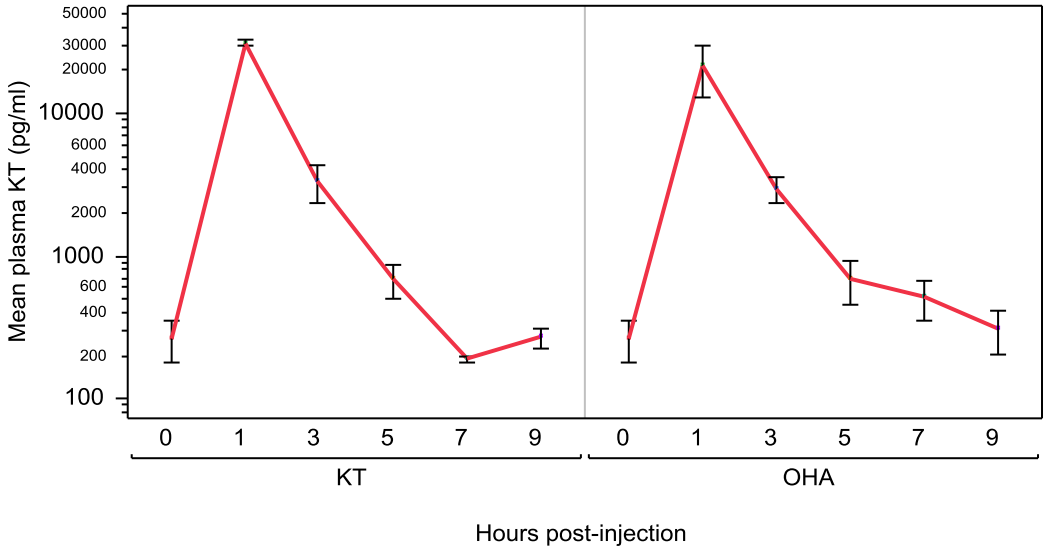


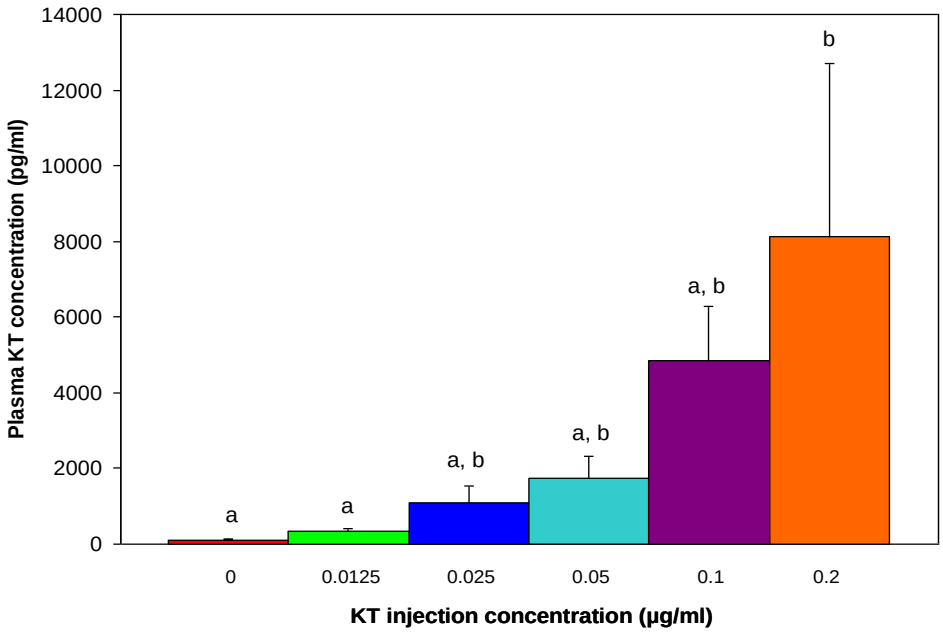

Fig. 4.2. Circulating KT levels were elevated one hour post-injection in KT and OHA treated female *S. scovelli* (KT: $X^2 = 15.0$, $p = 0.01$; OHA: $X^2 = 13.6$, $p = 0.02$). Plasma KT returned to near-baseline levels around five hours after the injection.

*Experiment 2*

When all treatment females assayed were analyzed, intraperitoneal injections of different KT doses resulted in different plasma KT concentrations (Wilcoxon rank sums: $X^2 = 45.1$, $p < 0.0001$; Fig. 4.3a). Post-hoc analyses revealed that only saline (0 µg/ml KT) and 0.0125 µg/ml KT treatments differed from the 0.2 µg/ml KT dose (Tukey HSD, saline: $p = 0.04$, 0.0125 µg/ml KT: $p = 0.048$). When only plasma KT levels that were on the standard curve of the assay were considered, plasma KT levels were higher with increasing doses of KT (Wilcoxon rank sums: $X^2 = 23.3$, $p < 0.0001$; Fig. 4.3b). Post-hoc analyses revealed that all groups but the 0.0125 µg/ml KT and 0.025 µg/ml KT doses differed (Tukey HSD, 0.0125 and 0.025 µg/ml KT: $p = 0.42$, for all other comparisons: $p < 0.05$).

An ANCOVA (full model: $R^2 = 0.29$, $F_{11,74} = 2.31$, $p = 0.02$; Fig. 4.4) revealed a significant main effect of size-adjusted stimulus behavior ($F_{1,74} = 16.1$, $p= 0.0002$) and a significant interaction of treatment and size-adjusted stimulus behavior ($F_{5,74} = 2.41$, $p = 0.046$) on test female competitive behavior. The main effect of treatment was not significant ($F_{5,74} = 0.33$, $p = 0.89$). The parameter estimates showed that test female competitive behavior was decreased in females that received high stimulus behavior from their partners in the 0.05 µg/ml KT treatment group (0.05 µg/ml KT*size-adjusted stimulus behavior: $t = -2.30$, $p = 0.02$). Test females that received high stimulus behavior showed a U-shaped dose-response in competitive behavior, where the middle dose of 0.05 µg/ml KT resulted in less test female competitive behavior than both the lowest and

A

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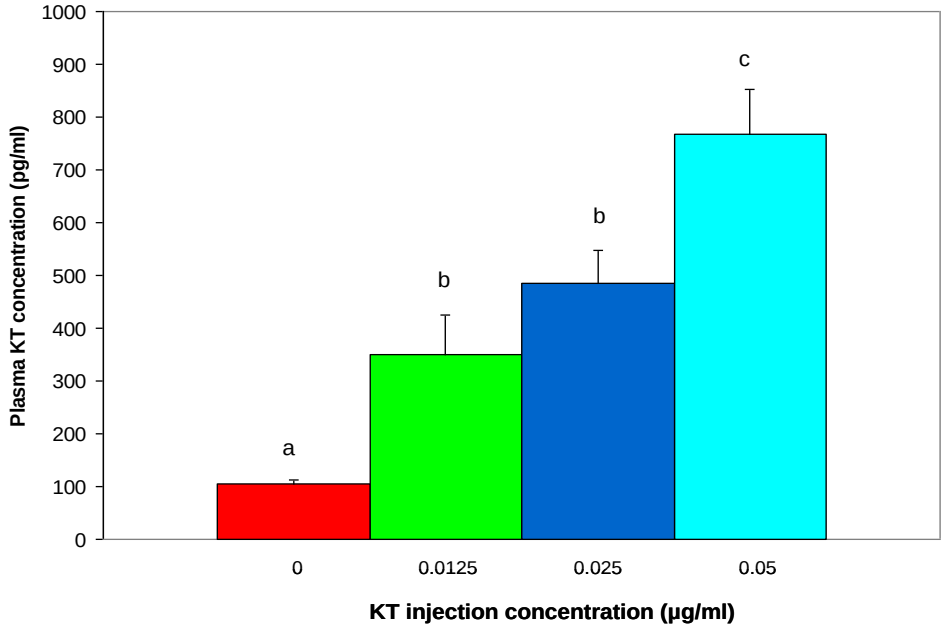


Fig. 4.3. Mean plasma KT concentration (pg/ml) for each treatment injection. (A) When all measured plasma samples were compared (n = 10 females in each group), only saline and 0.0125 µg/ml KT differed from the 0.2 µg/ml KT due to large variances in the higher doses. (B) When off-the-curve samples were removed from the analysis, most remaining treatment groups differed in the resultant plasma KT concentration (saline: n = 10, 0.0125 µg/ml KT: n = 10, 0.025 µg/ml KT: n = 7, 0.05 µg/ml KT: n = 6). Bars that do not share the same letter are significantly different. Error bars represent S.E.M.

highest doses of KT. A similar pattern of test female competitive behavior was seen for females that received low stimulus behavior, except for females in the 0.05 µg/ml KT treatment group; in this group, several females showed high levels of competitive behavior in response to receiving low stimulus behavior.

For test females that had physiological levels of circulating KT following treatment, a multiple linear regression analysis showed that the full model of test female KT, test female TL, stimulus female behavior, and their interactions had a significant effect on test female behavior (full model: $R^2 = 0.75$, $F_{7,17} = 4.24$, $p = 0.02$). The interactions of stimulus behavior*test TL and stimulus behavior*test KT were non-significant ($p > 0.92$ for both); these interactions were removed from the model and it was re-run. The reduced model (reduced model: $R^2 = 0.75$, $F_{7,17} = 7.12$, $p = 0.003$; Fig. 4.5) revealed a significant positive main effect of stimulus behavior ($F_{1,17} = 5.53$, $t = 2.35$, $p= 0.04$). However, the interactions of test TL*test KT (test TL*test KT: $F_{1,17} = 7.66$, $t = -2.77$, $p= 0.02$) and test TL*test KT* stimulus female behavior (test TL*test KT*stimulus behavior: $F_{1,17} = 10.02$, $t = -3.17$, $p= 0.008$) showed significant negative effects on test female competitive behavior. The main effects of test TL ($F_{1,17} = 0.08$, $t = 0.28$, $p = 0.77$) and test KT ($F_{1,17} = 0.13$, $t = 0.37$, $p = 0.72$) were not significant. These results suggest that the positive effect of increasing stimulus behavior on test female behavior is overridden by the negative interaction between test KT and test TL such that the three-way interaction of these variables produces an overall negative effect on test female competitive behavior.

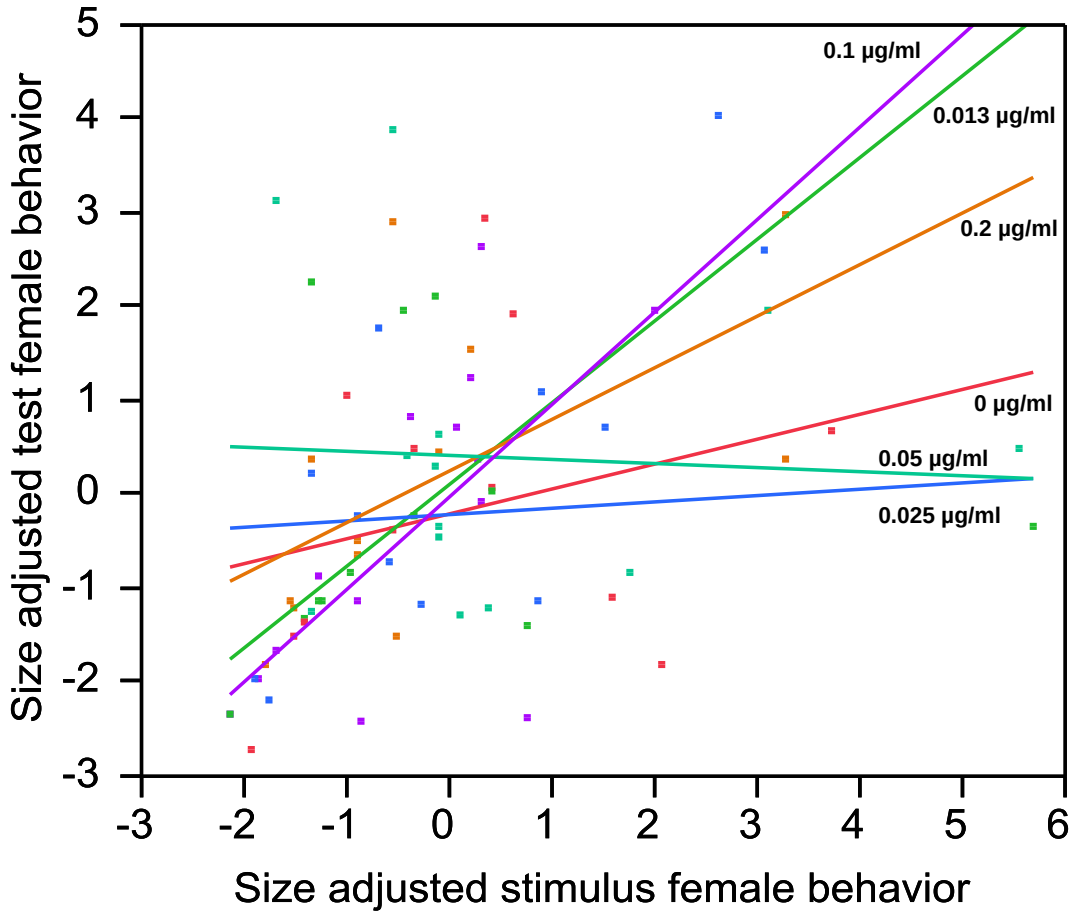


Fig. 4.4. The effect of test versus stimulus female behavior adjusted for total length for increasing doses of KT (ANCOVA, full model: $R^2 = 0.29$, $F_{11,74} = 2.31$, $p = 0.02$). Although most treatments have a positive slope of the regression line, the slope for the 0.05 µg/ml KT treatment group was significantly different than the control group (0 µg/ml KT) (0.05 µg/ml KT*size-adjusted stimulus behavior: $t = -2.30$, $p = 0.02$).

## Discussion

Ketotestosterone is the predominant plasma androgen in males of most fish species (Borg, 1994). It mediates a variety of male reproductive processes and behaviors (Goncalves and Oliveira, 2011). Comparatively little work has been done on the role of KT in female fish physiology and behavior (Oliveira and Goncalves, 2008). In this study, we aimed to determine how an acute elevation of KT affects the intrasexual competitive behavior of the sex-role reversed pipefish, *S. scovelli*. We first examined the time course of plasma KT elevation at 1, 3, 5, 7, and 9 hours following an injection of KT or OHA, an androgen that can be converted to KT in other teleosts (Kazeto *et al.*, 2011) and is the highest plasma androgen measured in *S. scovelli* (Scobell *et al.*, *In prep*). Next, we injected females with different doses of KT to determine whether increasing plasma levels of KT resulted in increasing levels of intrasexual female aggressive behavior. Lastly, we examined a subset of these females that had plasma KT levels within the physiological range to explore how natural fluctuations in KT might affect female behavior.

Intraperitoneal injections of both KT and OHA resulted in elevations of plasma KT. Peak circulating KT levels occurred one hour after the injection. Plasma KT levels at one hour were 80-fold greater than baseline levels in the OHA-injected group and 118-fold greater in the KT-injected group. These results suggest that both hormone solutions were absorbed into the circulation from the peritoneum and, in addition, that OHA was readily converted into KT in females. This conversion may have happened in the interrenal or

liver (Borg, 1994), in the ovary as has been demonstrated in the Anguilla eel (Kazeto *et al.*, 2011), or in the blood itself (Mayer et al., 1990; Schulz, 1986; Schulz and Blum, 1991). Plasma KT had returned to near-baseline levels three hours after the injection and remained low thereafter in both treatment groups. It can be inferred from this data that female *S. scovelli* can clear high concentrations of KT from the plasma in a relatively short amount of time.

We used the results from Experiment 1 to inform our experimental design for Experiment 2. We made serial dilutions of KT injection solutions with the highest dose being 1/10 that of the injection dose in Experiment 1. We began the competitive interaction trials one hour following the injection because the results from Experiment 1 suggested that we would achieve peak plasma KT concentrations around this time. Our injections did result in a dose-response type elevation of mean circulating KT levels in treatment females. However, many of the plasma KT values for the 0.1 and 0.2 µg/ml treatment doses were above the range of the standard curve and were extrapolated values. The large variances present in the higher doses obscured the differences in mean plasma concentration in the lower doses. When the off-the-curve samples were removed from the analysis, significant differences in plasma KT levels were observed between most of the remaining groups.

Contrary to our expectations, increasing doses of KT did not result in a linear increase in test female competitive behavior. Instead, there was an interaction between the dose of KT injected and the level of competitive behavior from the stimulus partner. Saline, low doses of KT, and high doses of KT resulted in a positive relationship

between stimulus and test female competitive behavior. This relationship was expected: if the stimulus female spends most of the trial resting in a corner of the tank, the test female usually does not show much competitive behavior, and conversely, if the stimulus female is very interactive, the test female has much more opportunity to show competitive behavior. What was not expected was the relationship observed in the 0.05 µg/ml KT treatment group. Test females that received low stimulus behavior from partners showed high competitive behavior themselves whereas test females that received high stimulus behavior from partners showed relatively low competitive behavior.

U-shaped dose-response curves are a common occurrence in hormone-behavior relationships. However, most curves are an inverted U-shape such that mid-range doses produce peak behavior. A U-shaped curve such as the one observed for females receiving high stimulus behavior are harder to interpret. It is possible that KT works through its native androgen receptor at low and mid-range doses, where increasing the dose of KT results in inhibition of competitive behavior. At pharmacological doses, as seen in our 0.1 and 0.2 µg/ml KT doses, KT concentrations might be activating a different steroid receptor with a much lower binding affinity for KT than the native receptor. At least two types of androgen receptors have been identified in teleost fish (Ikeuchi et al., 2001; Sperry and Thomas, 1999). In the Atlantic croaker (*Micropogonias undulates*), the two receptors have very different binding affinities for KT (Sperry and Thomas, 1999).

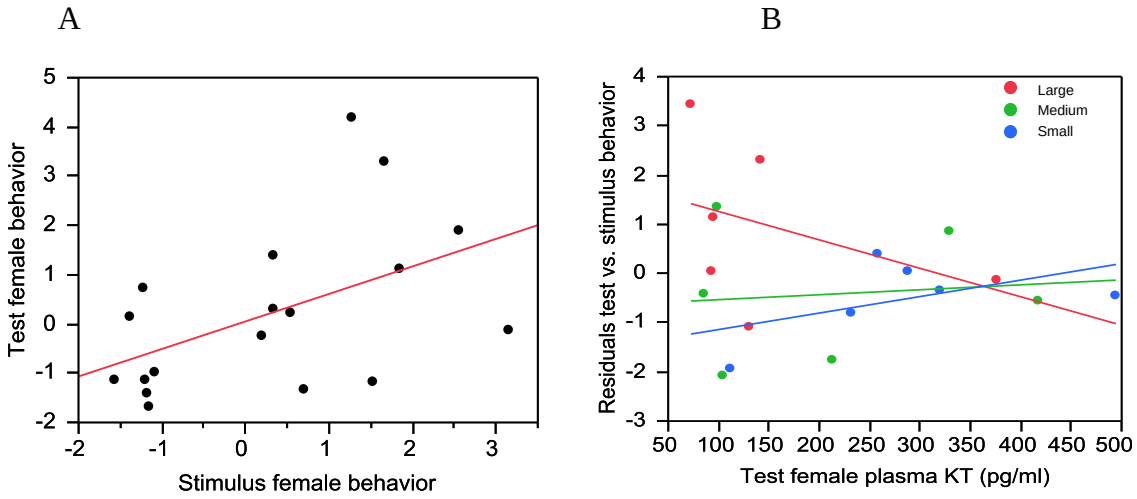


Fig. 4.5. Stimulus female behavior, test female KT, and test female TL interact to affect test female competitive behavior in females with physiological levels of KT (reduced model: $R^2 = 0.75$, $F_{7,17} = 7.12$, $p = 0.003$; test TL*test KT*stimulus behavior: $F_{1,17} = 10.02$, $p = 0.008$). (A) Females with physiological levels of KT showed an increase in competitive behavior when their partner showed more stimulus behavior ($F_{1,17} = 2.35$, $p = 0.04$). (B) When stimulus female behavior was controlled for, increasing levels of plasma KT had a greater negative effect on large test female competitive behavior (test TL*test KT: $F_{1,17} = 7.66$, $p = 0.02$).

When we examined the competitive behavior of test females that had circulating KT levels within the physiological range, we also found an overall negative effect of circulating KT level. Despite a positive relationship between stimulus and test female competitive behavior, the three-way interaction of test female circulating KT levels, test female total length, and stimulus female competitive behavior resulted in a negative effect of KT on test female competitive behavior. Thus, the negative interaction between test female circulating KT levels and test female total length overrode the positive effect of stimulus female behavior on test female competitive behavior. When we examined the effects of the interaction of test female circulating KT levels and test female total length, larger females showed more of a reduction in competitive behavior than did small or medium length females. It should be noted that the physiological upper limit we used was based on data from a population of *S. scovelli* collected in Sarasota, FL (Scobell *et al.*, *In prep*). The current study used fish collected off the coast of Texas. Fish from the Texas population were smaller (mean TL = 104.5 mm) than those from the Florida population (mean TL = 113.1 mm). The range of measured plasma KT levels from a field collection in females collected in Texas was 92.6 – 181.0 pg/ml (see Chapter II). It is possible that our injections of KT elevated plasma KT levels to a range that would be normal for the Florida population, but abnormal for the Texas population. If so, the decrease in behavior we observed could be due to supranormal physiological levels of KT and not reflect how KT modulates female competitive behavior in the field.

The results of the current study are opposite to those we found in our previous study (see Chapter III). In that study, larger females showed more competitive behavior than

small females with increasing plasma KT levels. There were two main methodological differences between these studies: the level of behavior from the stimulus female and the timing of the injection prior to the trial. In our previous study, stimulus females had been group-housed which resulted in social subordination and consequently, a low level of competitive interaction from these females. In the current study, stimulus females were housed individually in the same manner as test females, which lead to a greater variance in stimulus female behavior in the current study than in the previous study. Although the difference in stimulus female behavior between the two studies could account for the differences observed in test female behavior, we believe that the timing of the KT injection is more likely. In the previous study, we injected the KT solution the night before the trial, and in the current study, we injected the KT solution one hour before the trial. Considering the results of both studies, it is plausible that KT has a priming effect on female competitive behavior in *S. scovelli* in that prior exposure to KT facilitates the expression of aggressive behavior in subsequent interactions. The results of the current study make it seems much less likely that KT acts as a physiological trigger; higher doses of KT resulted in a greater inhibition of competitive behavior within the physiological range for KT in female *S. scovelli*. The latter synopsis may explain why the few species of syngnathids studied have lower plasma KT than OHA levels (Mayer *et al.*, 1993; Scobell *et al.*, *In prep*). If OHA acts as a readily available precursor to KT (but not as a biologically active androgen itself), females may use the high levels of circulating OHA to synthesize the small amounts of KT needed to prime the neuromuscular circuitry used in competitive behavior. In this way, *S. scovelli* females

could avoid the potential negative side effects of having consistently high levels of a more potent androgen like KT. This hypothesis requires further study because the results from Experiment 1 in this study suggest that females can readily convert large amounts of OHA to KT.

In both studies, large females appear to be more sensitive to the effects of KT on intrasexual competitive behavior than do small females. In the field, *S. scovelli* females that have mated with a male are larger, heavier, and more ornamented than females that had not successfully obtained a mate (Jones et al., 2001a). In the laboratory, males mate more quickly with larger females and produce a greater number of viable offspring from them (Paczolt and Jones, 2010). Male broadnose pipefish (*S. typhle*) and straightnose pipefish (*Nerophis ophidion*) also show a preference for larger females (Berglund *et al.*, 1986; Rosenqvist, 1990). These studies suggest that large body size is an important trait for obtaining a mate in sex-role reversed female syngnathids. Body size also seems to be an important trait in intrasexual competition. Larger pairs of interacting female *S. scovelli* showed more competitive behavior than smaller pairs (Scobell *et al.*, *In prep*). Larger *S. typhle* are more efficient at interrupting the courtship attempts of other females (Berglund, 1991). Larger females can also affect smaller females through dominance. When stimulus females were placed in a bottle in the test tank, female *S. typhle* mated with fewer males and transferred fewer eggs when a large female, but not a small female was visible (Berglund, 1991). The hormonal mechanisms mediating female intrasexual competition are likely to be more developed in large females that are more likely to engage in competitions than are small females. Smaller *S. scovelli* females may be

relatively insensitive to the behavioral effects of KT, whereas KT may facilitate competitive aggression in large females when acting in a priming capacity and inhibit competitive aggression during acute plasma increases.

In summary, we found that injections of either KT or OHA result in an increase in plasma KT concentrations that peak one hour following the injection. Different doses of KT interacted with stimulus female behavior to affect test female competitive behavior. The lowest and highest doses of KT resulted in a positive relationship between test and stimulus female competitive behavior whereas the mid-range dose resulted in a negative effect. When we examined females in the physiological range of circulating KT levels, stimulus female behavior had a positive effect on test female competitive behavior. However, the interaction between stimulus female behavior, test female total length, and test female plasma KT concentration had a negative effect on test female competitive behavior. These studies suggest that elevated plasma KT levels may be detrimental to the normal expression of female intrasexual competitive aggression in sex-role reversed *S. scovelli*.

# CHAPTER V

# CONCLUSIONS

The studies presented in this dissertation support a role for androgens in the regulation of male pregnancy and female intrasexual competitive behavior in the sex-role reversed Gulf pipefish, *Syngnathus scovelli*. In **CHAPTER I,** I reviewed the literature on endocrine studies in syngnathids. Studies in males suggested that androgens play a role in the development of the brood pouch during puberty and the regulation of spermatogenesis during adulthood. Androgen function is also likely integrated with that of prolactin and glucocorticoids to mediate male pregnancy. I constructed a model of the effects of androgens, prolactin, and glucocorticoids across the male reproductive cycle. In **CHAPTER II,** I tested this model with respect to the androgen 11-ketotestosterone in field-caught male and female Gulf pipefish. I found that males had significantly higher plasma levels of KT than females, despite the sex-role reversal of this species. Testis mass positively affected plasma KT levels in males. Both testis mass and plasma KT levels varied across the stages of pregnancy, suggesting that regulation of androgens is necessary for successful continuation of male pregnancy. In **CHAPTER III,** I examined the effects of KT, OHA, and cortisol on intrasexual competitive behavior in female Gulf pipefish. Despite OHA being the highest measured plasma androgen, it did not increase female competitive behavior. The effects of OHA were similar to those of cortisol, which disrupted the positive relationship between female body size and competitive behavior. Injections of KT resulted in a strengthening of the relationship between body size and competitive behavior such that large females performed more competitive

behavior than small females even though this androgen is the lowest plasma sex steroid measured in this species. In **CHAPTER IV,** I determined whether an acute plasma increase in KT would result in an increase in female intrasexual competitive behavior. The mid-range treatment dose resulted in a decrease in size-adjusted competitive behavior of test females when I controlled for size-adjusted competitive behavior of stimulus females. When only those females with plasma KT in the physiological range were considered, plasma KT level interacted with test female size and stimulus female behavior such that large females showed a greater decrease in competitive behavior than small females with increasing plasma KT.

Collectively, the results of this dissertation suggest that both male and female *S. scovelli* suffer reproductive consequences from inappropriate elevations of plasma KT: males suffer post-copulatory effects and females suffer pre-copulatory effects. If male *S. scovelli* have a similar physiological make-up as *H. hippocampus* and *H. guttulatus*, then we can expect that high circulating levels of KT during early to mid-pregnancy would result in a disruption of brooding and have negative impacts on embryos. In females, we showed the acute elevations of plasma KT resulted in a decrease in competitive behavior, which particularly impacted larger females. Considering the degree of similarity between the suite of behaviors that comprise competitive and courtship behavior, it is likely that abnormally elevated plasma KT levels would also negatively impact courtship behavior in *S. scovelli*. To avoid these negative effects of elevated KT, it appears that both sexes may modulate the expression of enzymes in the androgen synthesis pathway (Fig. 3.1) to synthesize KT at the right time and in the appropriate

amounts. In accordance with the results from our study in **CHAPTER II**, Mayer (1993) showed that *S. typhle* and *S. acus* males reduce plasma KT levels during brooding. The same pattern was observed for testosterone and 11β-hydroxytestosterone. However, plasma levels of OHA and ketoandrostenedione increased during brooding. These data suggest that males reduce the expression of the enzyme 17β-hydroxysteroid dehydrogenase (17β-HSD) during brooding. This would result in a decreased synthesis of testosterone and its metabolites, 11β-hydroxytestosterone and KT, from androstenedione and its metabolites, OHA and ketoandrostenedione, respectively, and explain the steroid hormone profile difference between breeding and brooding males. Females may also reduce the expression of 17β-HSD, and possibly 11β- hydroxysteroid dehydrogenase (11β-HSD) as well, to obtain low levels of circulating KT that can facilitate competitive behavior. Female *S. typhle* and *S. acus* showed a similar hormone profile to males in that plasma levels of testosterone, 11β-hydroxytestosterone, and KT were relatively lower than those of OHA and ketoandrostenedione (Mayer *et al.*, 1993). Female *S. scovelli* also had lower plasma levels of testosterone and KT than OHA (Scobell *et al., In prep*). Females may also up-regulate the enzyme aromatase, which synthesizes estradiol from testosterone, to ensure a sufficient circulating estradiol level necessary to maintain ovarian function. Despite low plasma levels of testosterone, females of all three Syngnathus species studied (*S. scovelli, S. acus, S. typhle*) have plasma estradiol levels similar to that of other female teleosts (Lokman et al., 2002b). Interestingly, female *S. scovelli* have a positive correlation between circulating KT and estradiol levels (Scobell *et al., In prep*), which supports the hypothesis that females

balance androgens and estrogens to mediate both reproductive function and sexual behavior in this species.

The accumulation of OHA, which may have little to no androgenic potency, would provide both males and females with a readily available source of androgen precursor to synthesize large amounts of KT when needed. For male syngnathids, it appears that spermatogenesis may take place over a short time period late in pregnancy. High plasma OHA (and/or ketoandrostenedione) would facilitate the rapid synthesis of KT needed to promote spermatogenesis over this period. In females, it is feasible that plasma KT levels increase with as oocytes mature as they do in Japanese eels (*Anguilla japonica*) (Kazeto *et al.*, 2011). For female syngnathids, the sequential nature of the asynchronous ovary suggests that the hormonal mediation of vitellogenesis, oocyte maturation, and ovulation must occur in a concerted fashion. Some syngnathids may have a very short period between the ovulation of each set of mature ova. In support of this prediction, female *S. scovelli* mated on average every 6.3 ± 0.8 days over a 61-day period (Scobell *et al.*, 2009). In *A. japonica*, OHA is a major steroid metabolite of oocytes; the production of OHA in oocytes peaks during mid-vitellogenesis and remains high thereafter (Kazeto *et al.*, 2011). The conversion rate of OHA to KT in *A. japonica* increases with advancing developmental stages of oocytes. If OHA and KT were synthesized in oocytes in *S. scovelli* as they are in *A. japonica*, we would predict a synthesis profile that reflects the nature of the asynchronous ovary. Plasma levels might reflect a continuous production of OHA from several cohorts of mid-vitellogenic oocytes punctuated by peaks of KT that are the product of the cohort of late-stage vitellogenic

oocytes in *S. scovelli*. We would expect the peaks of plasma KT to occur on a short time scale as each new cohort of oocytes reached the final stages of maturation. Future studies of KT in female *S. scovelli* should correlate KT levels with the stage of oogenesis in the most mature cohort of eggs or measure KT at progressive stages after mating to determine how KT levels correlate with ovulation. Comparative studies of the ovulatory cycle in pipefish and seahorses with differing types of ovaries (1 versus 2 germinal ridges) will also help determine whether physiology relates to the functional difference in mating pattern observed (polyandry/polygynandry versus monogamy).

Modulation of plasma KT levels may be a mechanism upon which natural selection can act to affect both pre- and post-copulatory sexual selection in syngnathids, with the ultimate result of the evolution of a new mating system. In the field, large, ornamented females have the highest mating success (Jones et al., 2001b). The results of **CHAPTER III** and **CHAPTER IV** suggest that large females are the most sensitive to the behavioral effects of KT. The disruption of courtship and competitive behavior by elevated KT could affect the mating dynamics within a population. The results of **CHAPTER II**, suggest that high plasma KT during early to mid-pregnancy are disruptive to normal embryo development. However, males may exploit this mechanism to exert post-copulatory sexual selection when mated with a non-preferred mate (Paczolt and Jones, 2010). One or both of these mechanisms could occur within a species. If male pregnancy is ancestral to female sex-role reversed behavior, it may be that the mechanism that modulates androgen synthesis in males to facilitate pregnancy is the same one that females access to mediate sex-role reversed behavior. Considering KT can

induce male sexual behavior in other teleosts (Kobayashi and Nakanishi, 1999; Semsar and Godwin, 2004; Stacey and Kobayashi, 1996) it is possible that KT was utilized first to induce a male-typical courtship behavior in females (syngnathid courtship is very intricate and coordination between males and females ensures the proper transfer of eggs). This behavior could have been co-opted for intrasexual competitive behavior by females in populations where competition for males was high. Given strong enough sexual selection, this mechanism could have evolved to produce female competitive behavior even in the absence of males, as is seen in *S. scovelli*.

**Future directions**

In spite of the technical challenges of conducting endocrinological studies with syngnathids, there exists sufficient endocrine data to suggest that further studies of hormone function will be fruitful in elucidating the physiological mechanisms regulating their unique reproductive biology. Future endocrine studies will require, however, the application of modern biochemical and molecular biological techniques capable of identifying hormones and characterizing hormone and receptor expression using very small quantities of tissue or fluids. Priority should be given to developing techniques capable of quantifying hormone concentrations in small volumes of biological fluids.

Sensitive immunoassays already exist for some hormones of interest, including reproductive and adrenal steroids and AVT. These assays should be validated in syngnathid species and then used to characterize circulating hormone changes, both in

the field and laboratory. Ideally, a mechanism for simultaneous detection of multiple hormones in small samples is likely to be most effective. Several techniques are currently being developed that measure multiple hormones from small volumes of plasma. Bykova and colleagues (2010) used capillary electrophoresis to measure eight steroids from 100-200 µl of plasma from yellow perch, *Perca flavescens*. This methodology is currently being modified to measure steroids in syngnathid plasma (J. Ripley, pers. comm.). Blasco and colleagues (2009) used high performance liquid chromatography followed by mass spectrometry (HPLC-MS) to measure T, KT and OHA from 50-100 µl of goldfish, *Carassius auratus*, plasma. Applying a similar methodology yet using ultra high performance liquid chromatography (UPLC-MS) would provide the sensitive limit of detection necessary to measure multiple hormones from smaller plasma volumes. Studies using techniques such as these are essential for identifying hormones most likely to influence gonadal development and reproductive behavior. However, studies using capillary electrophoresis or UPLC-MS would still not be amenable to repeated measurement, which is necessary to establish basal circulating levels of hormones within an individual during reproductive cycles. Measuring steroids from samples of fish holding water is a non-invasive way to assess hormone levels. This methodology is ideal for studies that use small and/or rare syngnathids (Scott and Ellis, 2007), and for behavior studies where multiple samples are required (Scott *et al.*, 2008). Kidd and colleagues (2010) measured multiple hormones (including T, KT, $E_2$, progesterone, and prostaglandin $F_{2\alpha}$) from a single water sample in *Astatotilapia burtoni* using commercial enzyme immunoassay (EIA) kits. Although hormone assays using

water sampling requires more validation than those that use plasma (Kidd et al., 2010; Scott et al., 2008), the accessibility and the non-invasive nature of this methodology make it a promising avenue for syngnathid hormone research. Once researchers develop standardized methodology for measuring hormone levels from water, plasma and other tissues, these techniques can be applied to common syngnathids to establish the parameters of normal reproductive cycles for males and females within a taxon.

Additional hormone administration studies are also needed to establish actions of steroid and protein hormones. In the case of steroid hormones, exposures can be simply achieved through immersion. Even some conserved peptide hormones can be applied with relative ease. For example, activation of the hypothalamo-pituitary-gonadal axis should be feasible through gonadotropin-releasing hormone administration, whereas mesotocin and AVT application may be very instructive in studying the regulation of ejaculation and parturition. However, studies of protein hormones will be more problematic, as previous injection studies utilizing mammalian hormones have raised species specificity and purity concerns.

Quantitative real-time PCR techniques for assessing hormone and receptor expression in the central nervous system, pituitary, gonad, and brood pouch should be developed as an extremely sensitive means to identify potential targets for hormones. Coupled with immunocytochemistry for receptor protein expression, these studies should begin to assess changes in target sensitivity to hormone stimulation throughout reproductive cycles. Such an approach is needed to expand our understanding of hormone function to additional potential players in the development and maintenance of

brood pouch function, such as growth hormone and IGF, expected to play a role in the tissue proliferation associated with brood pouch development and embryo implantation.

Although surgical manipulation remains a challenge in syngnathids, better husbandry techniques that reduce stressful environmental conditions should make recovery more likely and increase the success of such studies. Historically, rearing syngnathids in captivity was difficult and mortality was common due to problems with feeding and disease (Koldewey and Martin-Smith, 2010). Since the 1990s, studies of the life history and behavior of syngnathids (Berglund and Rosenqvist, 2003; Foster and Vincent, 2004) have helped make syngnathid aquaculture a viable prospect (Koldewey and Martin-Smith, 2010). Through the use of small-scale intensive systems (those with the most extreme levels of human control), enriched live and/or artificial feeds, and low animal densities, stress can be reduced and animals remain healthy (Koldewey and Martin-Smith, 2010). Although stress is clearly an issue for maintaining syngnathids in captivity, currently there have been only two studies on the stress response in seahorses (Anderson et al., 2011; Wright et al., 2007). Future endocrine studies in syngnathids should first examine basal corticosteroids under field and captive housing conditions as well as those elevated levels in response to a stressor. In addition to a better understanding of the syngnathid stress response, the challenge of extirpation-replacement studies may be partially overcome using hormone receptor agonists and antagonists. This approach was first addressed by Boisseau in 1967, but is currently underutilized in other syngnathid research.

Comprehensive studies of the hormonal regulation of reproduction in large, abundant “model” syngnathids should be pursued once these more sensitive techniques are broadly applicable. Researchers should determine how the main testicular androgens, testosterone and KT, affect brood pouch development during puberty and the regulation of spermatogenesis during the male reproductive cycle. Studies should aim to understand how these androgens interact with prolactin and glucocorticoids to regulate the brood pouch epithelium throughout pregnancy to promote proper embryo development. Future studies should identify which sex steroids are primarily synthesized by the gonads of both sexes during the reproductive cycle and of those present, which sex steroids are most important in reproduction. Further studies of how androgens and other hormones like AVT are involved in sex-role reversed behavior are needed. In addition, researchers should examine how endocrine disruptors affect syngnathid physiology. After the hormonal regulation of reproductive function and behavior is more clearly understood in these “model” syngnathids, researchers should conduct comparative studies that encompass the diversity of brood pouch complexity, sexual dimorphism, sexual behavior, and mating systems in the Family Syngnathidae. The ultimate goal of this line of research should be to determine how selection acts on reproductive structures and behavior to produce such novel traits as male pregnancy and sex-role reversal.

# REFERENCES

Alam, M.A., Komuro, H., Bhandari, R.K., Nakamura, S., Soyano, K., Nakamura, M., 2005. Immunohistochemical evidence identifying the site of androgen production in the ovary of the protogynous grouper *Epinephelus merra*. Cell Tiss. Res. 320, 323-329.

Anderson, P.A., Berzins, I.K., Fogarty, F., Hamlin, H.J., Guillette Jr, L.J., 2011. Sound, stress, and seahorses: The consequences of a noisy environment to animal health. Aquaculture 311, 129-138.

Andersson, M., 1994. Sexual Selection. Princeton University Press, Princeton, New Jersey.

Avise, J.C., Jones, A.G., Walker, D., Dewoody, J.A., Collaborators, 2002. Genetic mating systems and reproductive natural histories of fishes: Lessons for ecology and evolution. An. Rev. Gen. 36, 19-45.

Backström, T., Winberg, S., 2009. Arginine-vasotocin influence on aggressive behavior and dominance in rainbow trout. Phys. Behav. 96, 470-475.

Balment, R.J., Lu, W., Weybourne, E., Warne, J.M., 2006. Arginine vasotocin a key hormone in fish physiology and behaviour: A review with insights from mammalian models. Gen. Comp. Endocrinol. 147, 9-16.

Baron, D., Houlgatte, R., Fostier, A., Guiguen, Y., 2008a. Expression profiling of candidate genes during ovary-to-testis trans-differentiation in rainbow trout masculinized by androgens. Gen. Comp. Endocrinol. 156, 369-378.

Baron, D., Houlgatte, R., Fostier, A., Guiguen, Y., 2008b. Masculinization by 11 beta-hydroxyandrostenedione of female rainbow trout induces a marked dysregulation of gonadal gene expression profiles. Cybium 32, 98-98.

Baron, D., Montfort, J., Houlgatte, R., Fostier, A., Guiguen, Y., 2007. Androgen-induced masculinization in rainbow trout results in a marked dysregulation of early gonadal gene expression profiles. BMC Gen. 8, 357.

Begovac, P.C., Wallace, R.A., 1987. Ovary of the pipefish, *Syngnathus scovelli*. J. Morph. 193, 117-133.

Begovac, P.C., Wallace, R.A., 1988. Stages of oocyte development in the pipefish, *Syngnathus scovelli*. J. Morph. 197, 353-369.

Belanger, B., Fiet, J., Belanger, A., 1993. Effects of adrenocorticotropin on adrenal and plasma 11 beta-hydroxyandrostenedione in the guinea pig and determination of its relative androgen potency. Steroids 58, 29-34.

Ben-Jonathan, N., Hugo, E.R., Brandebourg, T.D., Lapensee, C.R., 2006. Focus on prolactin as a metabolic hormone. Trends Endocrinol. Metab. 17, 110-116.

Berglund, A., 1991. Egg Competition in a sex-role reversed pipefish: subdominant females trade reproduction for growth. Evolution 45, 770-774.

Berglund, A., 2000. Sex role reversal in a pipefish: female ornaments as amplifying handicaps. An. Zool. Fen. 37, 1-13.

Berglund, A., Rosenqvist, G., 2001a. Male pipefish prefer dominant over attractive females. Behav. Ecol. 12, 402-406.

Berglund, A., Rosenqvist, G., 2001b. Male pipefish prefer ornamented females. An. Behav. 61, 345-350.

Berglund, A., Rosenqvist, G., 2003. Sex role reversal in pipefish, in: Slater, P.J.B., Rosenblatt, J.S., Snowdon, C.T., Timothy, J.R. (Eds.), Advances in the Study of Behavior. Academic Press, pp. 131-167.

Berglund, A., Rosenqvist, G., Svensson, I., 1986. Mate choice, fecundity and sexual dimorphism in two pipefish (Syngathidae). Behav. Ecol. Sociobiol. 19, 301-307.

Bernet, P., Rosenqvist, G., Berglund, A., 1998. Female-female competition affects female ornamentation in the sex-role reversed pipefish *Syngnathus typhle*. Behaviour 135, 535-550.

Blasco, M., Carriquiriborde, P., Marino, D., Ronco, A.E., Somoza, G.M., 2009. A quantitative HPLC-MS method for the simultaneous determination of testosterone, 11-ketotestosterone and 11β-hydroxyandrostenedione in fish serum. J. Chromatog. B 877, 1509-1515.

Boisseau, J.P., 1965. Action de quelques hormones sur l'incubation d'hippocampes males normaux, ou castres, ou hypophysectomises. Comp. Rend. Hebdo. Sean. l'Acad. Sci. 260, 313–314.

Boisseau, J.P., 1967a. Les regulations hormonales de l'incubation chez un vertebre male: Recherches sur la reproduction de l'Hippocampe. PhD Thesis, University of Bordeaux, Bordeaux, France.

Boisseau, J.P., 1967b. Recherche sur le controle hormonal de l'incubation chez l'Hippocampe. Rev. Europ. d'Endocrinol. 4, 197–234

Boisseau, J.P., 1969. Prolactine et incubation chez l'hippocampe. Comp. Rend. l'Acad. Sci. Paris, Colloque International 177, 205–215.

Borg, B., 1994. Androgens in teleost fishes. Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. 109, 219-245.

Briffa, M., Sneddon, L.U., 2007. Physiological constraints on contest behaviour. Funct. Ecol. 21, 627-637.

Brown, J.D., 1972. A comparative life history study of four species of pipefishes (family Syngnathidae) in Florida. PhD dissertation, Univeristy of Florida, Gainesville, FL.

Bykova, L., Archer-Hartmann, S.A., Holland, L.A., Iwanowicz, L.R., Blazer, V.S., 2010. Steroid determination in fish plasma using capillary electrophoresis. Environ. Toxicol. Chem. 29, 1950-1956.

Carcupino, M., Baldacci, A., Corso, G., Franzoi, P., Pala, M., Mazzini, M., 1999. Testis structure and symplastic spermatid formation during spermatogenesis of pipefishes. J. Fish Biol. 55, 344-353.

Carcupino, M., Baldacci, A., Mazzini, M., Franzoi, P., 1997. Morphological organization of the male brood pouch epithelium of *Syngnathus abaster* Risso (Teleostea, Syngnathidae) before, during, and after egg incubation. Tiss. Cell 29, 21-30.

Carcupino, M., Baldacci, A., Mazzini, M., Franzoi, P., 2002. Functional significance of the male brood pouch in the reproductive strategies of pipefishes and seahorses: a

morphological and ultrastructural comparative study on three anatomically different pouches. J. Fish Biol. 61, 1465-1480.

Clotfelter, E.D., O'neal, D.M., Gaudioso, J.M., Casto, J.M., Parker-Renga, I.M., Snajdr, E.A., Duffy, D.L., Nolan, V., Jr., Ketterson, E.D., 2004. Consequences of elevating plasma testosterone in females of a socially monogamous songbird: evidence of constraints on male evolution? Horm. Behav. 46, 171-8.

Clutton-Brock, T.H., Vincent, A.C.J., 1991. Sexual selection and the potential reproductive rates of males and females. Nature 351, 58 - 60.

Consten, D., Keuning, E.D., Terlou, M., Lambert, J.G.D., Goos, H.J.T., 2002. Cortisol effects on the testicular androgen synthesizing capacity in common carp, *Cyprinus carpio* L. Fish Phys. Biochem. 25, 91-98.

Consten, D., Lambert, J.G., Goos, H.J., 2001. Cortisol affects testicular development in male common carp, *Cyprinus carpio* L., but not via an effect on LH secretion. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 129, 671-7.

De Waal, P.P., Wang, D.S., Nijenhuis, W.A., Schulz, R.W., Bogerd, J., 2008. Functional characterization and expression analysis of the androgen receptor in zebrafish (*Danio rerio*) testis. Reproduction 136, 225-234.

Denver, R.J., Hopkins, P.M., Mccormick, S.D., Propper, C.R., Riddiford, L., Sower, S.A., Wingfield, J.C., 2009. Comparative endocrinology in the 21st century. Integr. Comp. Biol. 49, 339-348.

Desjardins, J.K., Fernald, R.D., 2009. Fish sex: why so diverse? Curr. Op. Neurobiol. 19, 648-653.

Desjardins, J.K., Hazelden, M.R., Van Der Kraak, G.J., Balshine, S., 2006. Male and female cooperatively breeding fish provide support for the "Challenge Hypothesis". Behav. Ecol. 17, 149-154.

Desjardins, J.K., Stiver, K.A., Fitzpatrick, J.L., Milligan, N., Van Der Kraak, G.J., Balshine, S., 2008. Sex and status in a cooperative breeding fish: behavior and androgens. Behav. Ecol. and Sociobiol. 62, 785-794.

Desprez, D., Geraz, E., Hoarea, M.C., Melard, C., Bosc, P., Baroiller, J.F., 2003. Production of a high percentage of male offspring with a natural androgen, 11 beta-hydroxyandrostenedione (11 beta OHA4), in Florida red tilapia. Aquaculture 216, 55-65.

Dijkstra, P., Hekman, R., Schulz, R., Groothuis, T., 2007. Social stimulation, nuptial colouration, androgens and immunocompetence in a sexual dimorphic cichlid fish. Behav. Ecol. Sociobiol. 61, 599-609.

Dijkstra, P.D., Hemelrijk, C., Seehausen, O., Groothuis, T.G.G., 2009. Color polymorphism and intrasexual competition in assemblages of cichlid fish. Behav. Ecol. 20, 138-144.

Dloniak, S.M., French, J.A., Holekamp, K.E., 2006. Rank-related maternal effects of androgens on behaviour in wild spotted hyaenas. Nature 440, 1190-1193.

Ducrest, A.-L., Keller, L., Roulin, A., 2008. Pleiotropy in the melanocortin system, coloration and behavioural syndromes. Trends Ecol. Evol. 23, 502-510.

Dulka, J.G., Maler, L., 1994. Testosterone modulates female chirping behavior in the weakly electric fish, *Apteronotus leptorhynchus*. J. Comp. Physiol. A 174, 331-343.

Eens, M., Pinxten, R., 2000. Sex-role reversal in vertebrates: behavioural and endocrinological accounts. Behav. Proc. 51, 135-147.

Endo, T., Todo, T., Lokman, P.M., Kudo, H., Ijiri, S., Adachi, S., Yamauchi, K., 2010. Androgens and very low density lipoprotein are essential for the growth of previtellogenic oocytes from Japanese eel, *Anguilla japonica*, *in vitro*. Biol. Reprod. *In press.*

Fiedler, K., 1970. Hormonale auslösung der geburtsbewegungen beim seepferdchen (Hippocampus, Syngnathidae, Teleostei). Zeit. Tierpsychol. 27, 679-686.

Fitzpatrick, M.S., Gale, W.L., Schreck, C.B., 1994. Binding characteristics of an androgen receptor in the ovaries of coho salmon, *Oncorhynchus kisutch*. Gen. Comp. Endocrinol. 95, 399-408.

Fivizzani, A.J., Oring, L.W., 1986. Plasma steroid hormones in relation to behavioral sex role reversal in the spotted sandpiper, *Actitis macularia*. Biol. Reprod. 35, 1195-201.

Foran, C.M., Bass, A.H., 1999. Preoptic GnRH and AVT: axes for sexual plasticity in Teleost fish. Gen. Comp. Endocrinol. 116, 141-152.

Foster, S.J., Vincent, A.C.J., 2004. Life history and ecology of seahorses: implications for conservation and management. J. Fish Biol. 65, 1-61.

Godwin, J., 2010. Neuroendocrinology of sexual plasticity in teleost fishes. Front. Neuroendocrinol. 31, 203-216.

Goncalves, D.M., Oliveira, R.F., 2011. Hormones and sexual behavior of teleost fishes, in: Norris, D.O., Lopez, K.H. (Eds.), Hormones and Reproduction of Vertebrates. Amsterdam, Academic Press.

Goodson, J.L., Bass, A.H., 2000. Forebrain peptides modulate sexually polymorphic vocal circuitry. Nature 403, 769-772.

Goodson, J.L., Bass, A.H., 2001. Social behavior functions and related anatomical characteristics of vasotocin/vasopressin systems in vertebrates. Brain Res. Rev. 35, 246-265.

Govoroun, M., Mcmeel, O.M., D'cotta, H., Ricordel, M.J., Smith, T., Fostier, A., Guiguen, Y., 2001. Steroid enzyme gene expressions during natural and androgen-induced gonadal differentiation in the rainbow trout, *Oncorhynchus mykiss*. J. Exp. Zool. 290, 558-66.

Hattori, R.S., Fernandino, J.I., Kishii, A., Kimura, H., Kinno, T., Oura, M., Somoza, G.M., Yokota, M., Strussmann, C.A., Watanabe, S., 2009. Cortisol-induced masculinization: does thermal stress affect gonadal fate in pejerrey, a teleost fish with temperature-dependent sex determination? PLoS One 4, e6548.

Hausberger, M., Henry, L., Richard, M.A., 1995. Testosterone-induced Singing in Female European Starlings (*Sturnus vulgaris*). Ethology 99, 193-208.

Hayashi, Y., Kobira, H., Yamaguchi, T., Shiraishi, E., Yazawa, T., Hirai, T., Kamei, Y., Kitano, T., 2010. High temperature causes masculinization of genetically female medaka by elevation of cortisol. Mol. Reprod. Devel. 77, 679-86.

Hazon, N., Balment, R.J., 1998. Endocrinology, in: Evans, D.H. (Ed.), The Physiology of Fishes, 2nd Edition, CRC Press, Boca Raton, pp. 441-463.

Holownia, P., Owen, E.J., Conway, G.S., Round, J., Honour, J.W., 1992. Studies to confirm the source of 11 beta-hydroxyandrostenedione. J. Steroid Biochem. Mol. Biol. 41, 875-80.

Hutchinson, T.H., Brown, R., Brugger, K.E., Campbell, P.M., Holt, M., Lange, R., Mccahon, P., Tattersfield, L.J., Van Egmond, R., 2000. Ecological risk assessment of endocrine disruptors. Environ. Health Pers. 108, 1007-14.

Ikeuchi, T., Todo, T., Kobayashi, T., Nagahama, Y., 1999. cDNA cloning of a novel androgen receptor subtype. J. Biol. Chem. 274, 25205-25209.

Ikeuchi, T., Todo, T., Kobayashi, T., Nagahama, Y., 2001. Two subtypes of androgen and progestogen receptors in fish testes. Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. 129, 449-455.

Jones, A.G., Avise, J.C., 1997. Microsatellite analysis of maternity and the mating system in the Gulf pipefish *Syngnathus scovelli*, a species with male pregnancy and sex-role reversal. Mol. Ecol. 6, 203-13.

Jones, A.G., Avise, J.C., 2001a. Mating systems and sexual selection in male-pregnant pipefishes and seahorses: Insights from microsatellite-based studies of maternity. J. Hered. 92, 150-158.

Jones, A.G., Avise, J.C., 2001b. Mating systems and sexual selection in male-pregnant pipefishes and seahorses: insights from microsatellite-based studies of maternity. J. Hered. 92, 150-8.

Jones, A.G., Rosenqvist, G., Berglund, A., Avise, J.C., 1999. The genetic mating system of a sex-role-reversed pipefish (*Syngnathus typhle*): a molecular inquiry. Behav. Ecol. Sociobiol. 46, 357-365.

Jones, A.G., Walker, D., Avise, J.C., 2001. Genetic evidence for extreme polyandry and extraordinary sex-role reversal in a pipefish. Proc. R. Soc. B 268, 2531-2535.

Kawauchi, H., Sower, S.A., Moriyama, S., 2009. The Neuroendocrine Regulation of Prolactin and Somatolactin Secretion in Fish, in: Bernier, N.J., Kraak, G.V.D., Farrell, A.P., Brauner, C.J. (Eds.), Fish Physiology. Academic Press, London, pp. 197-234.

Kazeto, Y., Tosaka, R., Matsubara, H., Ijiri, S., Adachi, S., 2011. Ovarian steroidogenesis and the role of sex steroid hormones on ovarian growth and maturation of the Japanese eel. J. Steroid Biochem. Mol. Biol. 127, 149-154.

Kern, M.D., King, J.R., 1972. Testosterone-induced singing in female white-crowned sparrows. The Condor 74, 204-209.

Ketterson, E.D., 2007. Perspective. Mol. Ecol. 16, 1345-7.

Ketterson, E.D., Atwell, J.W., Mcglothlin, J.W., 2009. Phenotypic integration and independence: Hormones, performance, and response to environmental change. Integr. Comp. Biol. 49, 365-79.

Khan, M.N., Reddy, P.K., Renaud, R.L., Leatherland, J.F., 1997. Application of HPLC methods to identify plasma profiles of 11-oxygenated androgens and other steroids in arctic charr (*Salvelinus alpinus*) during gonadal recrudescence. Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. 118, 221-227.

Kidd, C.E., Kidd, M.R., Hofmann, H.A., 2010. Measuring multiple hormones from a single water sample using enzyme immunoassays. Gen. Comp. Endocrinol. 165, 277-285.

Knapp, R., Carlisle, S.L., 2011a. Testicular function and hormonal regulation in fishes, in: Norris, D.O., Lopez, K.L. (Eds.), Hormones in Vertebrates. Academic Press, Amsterdam.

Knapp, R., Wingfield, J.C., Bass, A.H., 1999. Steroid hormones and paternal care in the plainfin midshipman fish (*Porichthys notatus*). Horm. Behav. 35, 81-89.

Kobayashi, M., Nakanishi, T., 1999. 11-Ketotestosterone induces male-type sexual behavior and gonadotropin secretion in gynogenetic crucian carp, *Carassius auratus langsdorfii*. Gen. Comp. Endocrinol. 115, 178-187.

Koldewey, H.J., Martin-Smith, K.M., 2010. A global review of seahorse aquaculture. Aquaculture 302, 131-152.

Kornienko, E.S., 2001. Reproduction and development in some genera of pipefish and seahorses of the Family Syngnathidae. Rus. J. Mar. Biol. 27, S15-S26.

Kornienko, E.S., Drosdov, A.L., 1999. Gametogenesis in the far Eastern pipefish *Syngnathus acusimilis*. Rus. J. Mar. Biol. 25, 353-357.

Kortner, T.M., Rocha, E., Arukwe, A., 2009. Androgenic modulation of early growth of Atlantic cod (*Gadus morhua* L.) previtellogenic oocytes and zona radiata-related genes. J. Toxicol. Environ. Health A 72, 184-95.

Kortner, T.M., Rocha, E., Silva, P., Castro, L.F., Arukwe, A., 2008. Genomic approach in evaluating the role of androgens on the growth of Atlantic cod (*Gadus morhua*) previtellogenic oocytes. Comp. Biochem. Physiol. D: Genom. Proteom. 3, 205-18.

Kujala, G.A., 1978. Corticosteroid and neurohypophyseal hormone control of parturition in the guppy, *Poecilia reticulata*. Gen. Comp. Endocrinol. 36, 286-296.

Kurtz, J., Kalbe, M., Langefors, A., Mayer, I., Milinski, M., Hasselquist, D., 2007. An experimental test of the immunocompetence handicap hypothesis in a teleost fish: 11-ketotestosterone suppresses innate immunity in three-spined sticklebacks. Am. Nat. 170, 509-19.

Kuzminski, H., Dobosz, S., 2010. Effect of sex reversal in rainbow trout (*Oncorhynchus mykiss* Walbaum) using 17α-methyltestosterone and 11β-hydroxyandrostenedione. Arch. Pol. Fish. 18, 45-49.

Kvarnemo, C., Moore, G.I., Jones, A.G., Nelson, W.S., Avise, J.C., 2000. Monogamous pair bonds and mate switching in the Western Australian seahorse *Hippocampus subelongatus*. J. Evol. Biol. 13, 882-888.

Kvarnemo, C., Simmons, L.W., 2004. Testes investment and spawning mode in pipefishes and seahorses (Syngnathidae). Biol. J. Linn. Soc. 83, 369-376.

Langmore, N.E., Cockrem, J.F., Candy, E.J., 2002. Competition for male reproductive investment elevates testosterone levels in female dunnocks, *Prunella modularis.* Proc. R. Soc. B 269, 2473-8.

Lema, S.C., Nevitt, G.A., 2004. Exogenous vasotocin alters aggression during agonistic exchanges in male Amargosa River pupfish (*Cyprinodon nevadensis amargosae*). Horm. Behav. 46, 628-37.

Lockwood, S., 1867. The sea-horse and its young. Am. Nat. 1, 225-234.

Lokman, P.M., George, K.A., Divers, S.L., Algie, M., Young, G., 2007. 11-Ketotestosterone and IGF-I increase the size of previtellogenic oocytes from shortfinned eel, *Anguilla australis*, *in vitro*. Reproduction 133, 955-67.

Lokman, P.M., Harris, B., Kusakabe, M., Kime, D.E., Schulz, R.W., Adachi, S., Young, G., 2002. 11-Oxygenated androgens in female teleosts: prevalence, abundance, and life history implications. Gen. Comp. Endocrinol. 129, 1-12.

Lubzens, E., Young, G., Bobe, J., Cerdà, J., 2010. Oogenesis in teleosts: How fish eggs are formed. Gen. Comp. Endocrinol. 165, 367-389.

Mank, J.E., 2007. The evolution of sexually selected traits and antagonistic androgen expression in actinopterygiian fishes. Am Nat 169, 142-9.

Mayer, I., Borg, B., Pall, M., 2004. Hormonal control of male reproductive behaviour in fishes: A stickleback perspective. Behavior 141, 1499-1510.

Mayer, I., Borg, B., Schulz, R., 1990. Conversion of 11-ketoandrostenedione to 11-ketotestosterone by blood cells of six fish species. Gen. Comp. Endocrinol. 77, 70-4.

Mayer, I., Rosenqvist, G., Borg, B., Ahnesjo, I., Berglund, A., Schulz, R.W., 1993. Plasma levels of sex steroids in three species of pipefish (Syngnathidae). Can. J. Zool. 71, 1903-1907.

Mccarthy, M.M., 2008. Estradiol and the developing brain. Phys. Rev. 88, 91-134.

Mccoy, E.E., Jones, A.G., Avise, J.C., 2001. The genetic mating system and tests for cuckoldry in a pipefish species in which males fertilize eggs and brood offspring externally. Mol. Ecol. 10, 1793-1800.

Mcglothlin, J.W., Neudorf, D.L., Casto, J.M., Nolan, V., Jr., Ketterson, E.D., 2004. Elevated testosterone reduces choosiness in female dark-eyed juncos (*Junco hyemalis*): evidence for a hormonal constraint on sexual selection? Proc. Biol. Sci. B 271, 1377-84.

Miura, T., Yamauchi, K., Takahashi, H., Nagahama, Y., 1991. Hormonal induction of all stages of spermatogenesis in vitro in the male Japanese eel (*Anguilla japonica*). Proc. Nat. Acad. Sci. 88, 5774-5778.

Munakata, A., Kobayashi, M., 2010. Endocrine control of sexual behavior in teleost fish. Gen. Comp. Endocrinol. 165, 456-468.

Munro, A.D., Pitcher, T.J., 1985. Steroid hormones and agonistic behavior in a cichlid teleost, *Aequidens pulcher*. Horm. Behav. 19, 353-371.

Mylonas, C.C., Zohar, Y., 2001. Use of GnRHa-delivery systems for the control of reproduction in fish. Rev. Fish Biol. Fisher. 10, 463-491.

Nelson, R.J., 2000. An Introduction to Behavioral Endocrinology, Second Edition ed. Sinauer Associates, Inc., Sunderland, MA.

Nelson, R.J., 2005. An Introduction to Behavioral Endocrinology, Third Edition ed. Sinauer Associates, Inc., Sunderland, MA.

Norris, D.O., 2007. Vertebrate Endocrinology, 4th ed. Academic Press, Amsterdam.

Noumura, T., 1959. Induction of marsupium-like structure by testosterone in females of the pipefish, Syngnathus schlegeli. J. Fac. Sci.. University of Tokyo IV, 515-520.

Oliveira, R.F., Goncalves, D.M., 2008. Hormones and social behavior of teleost fish, in: Magnhagen;, C., Braithwaite, V.A., Forsgren, E., Kapoor, B.G. (Eds.), Fish Behavior. Science Publishers, Enfield (NH), pp. 61-150.

Oliveira, R.F., Hirschenhauser, K., Carneiro, L.A., Canario, A.V.M., 2002. Social modulation of androgen levels in male teleost fish. Comp Biochem Physiol B Biochem Mol Biol 132, 203-215.

Paczolt, K.A., Jones, A.G., 2010. Post-copulatory sexual selection and sexual conflict in the evolution of male pregnancy. Nature 464, 401-404.

Pall, M.K., Liljander, M., Borg, B., 2004. Prolactin diminishes courtship behaviour and stimulates fanning in nesting male three-spined sticklebacks, *Gasterosteus aculeatus*. Behavior 141, 1511-1519.

Pall, M.K., Mayer, I., Borg, B., 2002. Androgen and behavior in the male three-spined stickleback, *Gasterosteus aculeatus*. II. Castration and 11-ketoandrostenedione effects on courtship and parental care during the nesting cycle. Horm. Behav. 42, 337-344.

Partridge, C., Boettcher, A., Jones, A.G., 2010. Short-term exposure to a synthetic estrogen disrupts mating dynamics in a pipefish. Horm. Behav. 58, 800-807.

Partridge, C., Shardo, J., Boettcher, A., 2007. Osmoregulatory role of the brood pouch in the euryhaline Gulf pipefish, *Syngnathus scovelli*. Comp. Biochem. Physiol. A 147, 556-61.

Patron, J.J., Herrera, A.A., Oconer, E.P., 2008. Prolactin and growth hormone levels in the pouch fluid of gravid male seahorse, *Hipocampus barbouri* Jordan and Richardson 1908. Asia Life Sci. 17, 261-269.

Peute, J., Schulz, R., Glazenburg, K., Lambert, J.G., Blum, V., 1989. Pituitary steroids in two teleost species: immunohistological and biochemical studies. Gen. Comp. Endocrinol. 76, 63-72.

Poortenaar, C.W., Woods, C.M.C., James, P.J., Giambartolomei, F.M., Lokman, P.M., 2004. Reproductive biology of female big-bellied seahorses. J. Fish Biol. 64, 717-725.

Reddy, P.K., Renaud, R., Leatherland, J.F., 1999. Effects of cortisol and triiodo-L-thyronine on the steroidogenic capacity of rainbow trout ovarian follicles at two stages of oocyte maturation. Fish Phys. Biochem. 21, 129-140.

Reid, G.K., 1954. An ecological study of the Gulf of Mexico fishes, in the vicinity of Cedar Key, Florida. Bull. Mar. Sci. Gulf Carib. 4, 1-94.

Ripley, J.L., 2009. Osmoregulatory role of the paternal brood pouch for two Syngnathus species. Comp. Biochem. Physiol. A 154, 98-104.

Ripley, J.L., Foran, C., 2010. Elevated whole brain arginine vasotocin with Aroclor 1254 exposure in two *Syngnathus* pipefishes. Fish Phys. Biochem. 36, 917-921.

Ripley, J.L., Foran, C.M., 2009a. Direct evidence for embryonic uptake of paternally-derived nutrients in two pipefishes (Syngnathidae: *Syngnathus* spp.). J. Comp. Physiol. B 179, 325-33.

Ripley, J.L., Foran, C.M., 2009b. Quantification of whole brain arginine vasotocin for two Syngnathus pipefishes: elevated concentrations correlated with paternal brooding. Fish Physiol. Biochem. 36, 867–874.

Rosenqvist, G., 1990. Male mate choice and female-female competition for mates in the pipefish *Nerophis ophidion*. Anim. Behav. 39, 1110-1115.

Rutkowska, J., Cichon, M., Puerta, M., Gil, D., 2005. Negative effects of elevated testosterone on female fecundity in zebra finches. Horm. Behav. 47, 585-91.

Sakamoto, T., Mccormick, S.D., 2006. Prolactin and growth hormone in fish osmoregulation. Gen. Comp. Endocrinol. 147, 24-30.

Schulz, R., 1986. In vitro metabolism of steroid hormones in the liver and in blood cells of male rainbow trout (*Salmo gairdneri* Richardson). Gen. Comp. Endocrinol. 64, 312-9.

Schulz, R., Blum, V., 1991. Extragonadal 17 beta-hydroxysteroid dehydrogenase activity in rainbow trout. Gen. Comp. Endocrinol. 82, 197-205.

Schulz, R., Miura, T., 2002. Spermatogenesis and its endocrine regulation. Fish Phys. Biochem. 26, 43-56.

Schulz, R.W., De França, L.R., Lareyre, J.-J., Legac, F., Chiarini-Garcia, H., Nobrega, R.H., Miura, T., 2010. Spermatogenesis in fish. Gen. Comp. Endocrinol. 165, 390-411.

Scobell, S.K., Fudickar, A.M., Knapp, R., 2009. Potential reproductive rate of a sex-role reversed pipefish over several bouts of mating. Anim. Behav. 78, 747-753.

Scobell, S.K., Mackenzie, D.S., 2011. Reproductive endocrinology of Syngnathidae. J. Fish Biol. 78, 1662-1680.

Scobell, S.K., Rosenthal, G.G., Knapp, R., *In prep*. Winning females have higher levels of 11β-hydroxyandrostenedione in a sex-role reversed pipefish.

Scott, A.P., Ellis, T., 2007. Measurement of fish steroids in water--a review. Gen. Comp. Endocrinol. 153, 392-400.

Scott, A.P., Hirschenhauser, K., Bender, N., Oliveira, R., Earley, R.L., Sebire, M., Ellis, T., Pavlidis, M., Hubbard, P.C., Huertas, M., Canario, A., 2008. Non-invasive measurement of steroids in fish-holding water: important considerations when applying the procedure to behaviour studies. Behaviour 145, 1307-1328.

Selman, K., Wallace, R.A., Player, D., 1991. Ovary of the seahorse, *Hippocampus erectus*. J. Morph. 209, 285-304.

Semenkova, T.B., Canário, A.V.M., Bayunova, L.V., Couto, E., Kolmakov, N.N., Barannikova, I.A., 2006. Sex steroids and oocyte maturation in the sterlet (*Acipenser ruthenus* L.). J. App. Ichthyol. 22, 340-345.

Semsar, K., Godwin, J., 2003. Social influences on the arginine vasotocin system are independent of gonads in a sex-changing fish. J. Neurosci. 23, 4386-4393.

Semsar, K., Godwin, J., 2004. Multiple mechanisms of phenotype development in the bluehead wrasse. Horm. Behav. 45, 345-353.

Semsar, K., Kandel, F.L.M., Godwin, J., 2001. Manipulations of the AVT system shift social status and related courtship and aggressive behavior in the bluehead wrasse. Horm. Behav. 40, 21-31.

Serkov, V., Kornienko, M., Kolobov, V., 2007. Structural and functional features of gill epithelium and the brood pouch of pipefish *Sygnathus acusimilis* (Syngnathidae, Gasterosteiformes) during adaptation to dilute sea water. J. Ichthyol. 47, 750-754.

Sköld, H.N., Amundsen, T., Svensson, P.A., Mayer, I., Bjelvenmark, J., Forsgren, E., 2008. Hormonal regulation of female nuptial coloration in a fish. Horm. Behav. 54, 549-556.

Sogabe, A., Matsumoto, K., Ohashi, M., Watanabe, A., Takata, H., Murakami, Y., Omori, K., Yanagisawa, Y., 2008. A monogamous pipefish has the same type of ovary as observed in monogamous seahorses. Biol. Lett. 4, 362-5.

Sperry, T.S., Thomas, P., 1999. Characterization of two nuclear androgen receptors in Atlantic croaker: Comparison of their biochemical properties and binding specificities. Endocrinology 140, 1602-1611.

Stacey, N., 2011. Hormonally derived sex pheromones in fishes, in: Norris, D.O., Lopez, K.H. (Eds.), Hormones in Vertebrates. Academic Press, Amsterdam.

Stacey, N., Kobayashi, M., 1996. Androgen induction of male sexual behaviors in female goldfish. Horm. Behav. 30, 434-445.

Staub, N.L., De Beer, M., 1997. The Role of androgens in female vertebrates. Gen. Comp. Endocrinol. 108, 1-24.

Stockley, P., Gage, M.J.G., Parker, G.A., Moller, A.P., 1996. Female reproductive biology and the coevolution of ejaculate characteristics in fish. Proc. R. Soc. B 263, 451-458.

Stölting, K.N., Wilson, A.B., 2007. Male pregnancy in seahorses and pipefish: beyond the mammalian model. Bioessays 29, 884-96.

Suzuki, Y., Nakagawa, M., Sato, F., Iichikawa, Y., Mizushima, Y., 2000. A primary adrenal steroid, 11 beta-hydroxyandrostenedione, has an osteotropic effect and little androgenic activity. J. Steroid Biochem. Mol. Biol. 74, 203-211.

Taves, M.D., Desjardins, J.K., Mishra, S., Balshine, S., 2009. Androgens and dominance: Sex-specific patterns in a highly social fish (*Neolamprologus pulcher*). Gen. Comp. Endocrinol. 161, 202-207.

Ueda, N., Partridge, C., Bolland, J., Hemming, J., Sherman, T., Boettcher, A., 2005. Effects of an environmental estrogen on male gulf pipefish, *Syngnathus scovelli* (Evermann and Kendall), a male brooding teleost. Bull. Environ. Contam. Toxicol. 74, 1207-12.

Urbatzka, R., Rocha, M.J., Rocha, E., 2011. Regulation of ovarian development and function in teleosts, in: Norris, D.O., Lopez, K.H. (Eds.), Hormones and Reproduction in Vertebrates. Academic Press, Amsterdam.

Van Look, K.J., Dzyuba, B., Cliffe, A., Koldewey, H.J., Holt, W.V., 2007. Dimorphic sperm and the unlikely route to fertilisation in the yellow seahorse. J. Exp. Biol. 210, 432-7.

Vincent, A., Ahnesjö, I., Berglund, A., Rosenqvist, G., 1992. Pipefishes and seahorses: Are they all sex role reversed? Trends Ecol. Evol 7, 237-241.

Vincent, A.C.J., 1994. Seahorses exhibit conventional sex roles in mating competition, despite male pregnancy. Behaviour 128, 135-151.

Vincent, A.C.J., Berglund, A., Ahnesjö, I., 1995. Reproductive ecology of five pipefish species in one eelgrass meadow. Environ. Biol. Fishes 44, 347-361.

Vizziano, D., Baron, D., Randuineau, G., Mahe, S., Cauty, C., Guiguen, Y., 2008. Rainbow trout gonadal masculinization induced by inhibition of estrogen synthesis is more physiological than masculinization induced by androgen supplementation. Biol. Reprod. 78, 939-46.

Von Engelhardt, N., Kappeler, P.M., Heistermann, M., 2000. Androgen levels and female social dominance in *Lemur catta*. Proc. Biol. Sci. B 267, 1533-9.

Watanabe, S., Hara, M., Watanabe, Y., 2000. Male internal fertilization and introsperm-like sperm of the seaweed pipefish (*Syngnathus schlegeli*). Zool. Sci. 17, 759-767.

Watanabe, S., Kaneko, T., Watanabe, Y., 1999. Immunocytochemical detection of mitochondria-rich cells in the brood pouch epithelium of the pipefish, *Syngnathus schlegeli*: structural comparison with mitochondria-rich cells in the gills and larval epidermis. Cell Tissue Res. 295, 141-9.

Watson, J.T., Kelley, D.B., 1992. Testicular masculinization of vocal behavior in juvenile female *Xenopus laevis* reveals sensitive periods for song duration, rate, and frequency spectra. J. Comp. Phys. A 171, 343-350.

Wilson, A.B., 2006. Interspecies mating in sympatric species of *Syngnathus pipefish*. Mol. Ecol. 15, 809-24.

Wilson, A.B., Ahnesjo, I., Vincent, A.C., Meyer, A., 2003. The dynamics of male brooding, mating patterns, and sex roles in pipefishes and seahorses (family Syngnathidae). Evolution 57, 1374-86.

Wilson, A.B., Martin-Smith, K.M., 2007. Genetic monogamy despite social promiscuity in the pot-bellied seahorse (*Hippocampus abdominalis*). Mol. Ecol. 16, 2345-52.

Wilson, A.B., Vincent, A., Ahnesjö, I., Meyer, A., 2001. Male pregnancy in seahorses and pipefishes (Family Syngnathidae): Rapid diversification of paternal brood pouch morphology inferred from a molecular phylogeny. J. Hered. 92, 159-166.

Wingfield, J.C., Jacobs, J., Hillgarth, N., 1997. Ecological constraints and the evolution of hormone-behavior interrelationships. An. N.Y. Acad. Sci. 807, 22-41.

Woodley, S.K., Moore, M.C., 1999. Ovarian hormones influence territorial aggression in free-living female mountain spiny lizards. Horm. Behav. 35, 205-14.

Wright, K.A., Woods, C.M.C., Gray, B.E., Lokman, P.M., 2007. Recovery from acute, chronic and transport stress in the pot-bellied seahorse *Hippocampus abdominalis*. J. Fish Biol. 70, 1447-1457.

Yamaguchi, T., Yoshinaga, N., Yazawa, T., Gen, K., Kitano, T., 2010. Cortisol is involved in temperature-dependent sex determination in the Japanese flounder. Endocrinology 151, 3900-8.

Yanase, M., Gorski, R.A., 1976. Sites of estrogen and progesterone facilitation of lordosis behavior in the spayed rat. Biol. Reprod. 15, 536-543.

Ziegler, T.E., 2000. Hormones associated with non-maternal infant care: a review of mammalian and avian studies. Folia Prim. 71, 6-21.

Zohar, Y., Muñoz-Cueto, J.A., Elizur, A., Kah, O., 2010. Neuroendocrinology of reproduction in teleost fish. Gen. Comp. Endocrinol. 165, 438-455.

Zysling, D.A., Greives, T.J., Breuner, C.W., Casto, J.M., Demas, G.E., Ketterson, E.D., 2006. Behavioral and physiological responses to experimentally elevated testosterone in female dark-eyed juncos (*Junco hyemalis* carolinensis). Horm. Behav. 50, 200-7.

# VITA

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## EDUCATION

| | |
|---|---|
| Aug. 2006 –Dec. 2011 | **Ph.D. Student** (Biology), Texas A&M University, College Station<br>(Advisors: Adam G. Jones, Gil G. Rosenthal) |
| August 2006 | **M.S.** (Zoology), University of Oklahoma, Norman<br>Thesis: Potential reproductive rate and female aggression in the sex-role reversed Gulf pipefish, *Syngnathus scovelli* (Advisor: Rosemary Knapp) |
| May 1999 | **B.A.** (Biology), *Cum laude*, Augustana College, Rock Island, Illinois |

## RESEARCH INTERESTS

Evolution of Mating Systems, Evolution of Sex-role Reversal, Molecular Biology, Endocrinology

## PROFESSIONAL EXPERIENCE

| | |
|---|---|
| Fall 2009-Fall 2011 | *Graduate Teaching Assistant*, Department of Biology, Texas A&M University<br>Courses: Human Anatomy and Physiology |
| June 2008 – 2009 | *Graduate Fellow*, National Science Foundation GK-12 Fellows Program, Partnership for Environmental Education and Rural Health, Texas A&M University, College Station |
| Fall 2006 – 2007 | *Graduate Trainee*, Molecular and Cellular Biology Training Program, Department of Biochemistry and Biophysics and Department of Biology, Texas A&M University, College Station |

## HONORS AND AWARDS

| | |
|---|---|
| 2005-2006 | Adams Memorial Scholarship, Dept.of Zoology, Univ. of Oklahoma |

## GRANTS RECEIVED

| | |
|---|---|
| 2011 | Interdisciplinary Faculty of Reproductive Biology Graduate Student Travel Award, $1100 |

## PUBLICATIONS

Scobell SK, Fudickar AM, Knapp R. 2009. Potential reproductive rate of a sex-role reversed pipefish over several bouts of mating. *Animal Behavior* 78: 747–753.

Scobell SK and MacKenzie DS. 2011. Reproductive endocrinology of syngnathids. J. Fish Biol. 78: 1662-1680.

## SCIENTIFIC MEMBERSHIPS

American Association for the Advancement of Science
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